MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* AND *STAPHYLOCOCCUS EPIDERMIDIS* STRAINS STEPWISE ADAPTED TO VANCOMYCIN

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NOMENCLATURE

1. *S. aureus*: gram positive cocci arranged in grape-like clusters. Can be normal flora. Common pathogen of skin, trauma, lungs. Biochemical reactions include coagulase and mannitol salts positive
2. S. *epidermidis*: gram positive cocci arranged in clusters. Normal skin flora. Opportunistic pathogen. Coagulase and mannitol salts non-fermenter.
3. *Enterococcus*: gram positive cocci in chains. Typically normal intestinal flora. May be pathogenic.
4. Antimicrobials: compounds which inhibit the growth and/or reproduction of microorganisms.
5. Beta-lactams: antimicrobials with β-lactam ring structure which inhibits microbial cell wall synthesis. Examples are penicillins and cephalosporins.
6. Vancomycin: glycopeptide antimicrobial that inhibits cell wall synthesis.
7. MRSA: methicillin–resistant *S. aureus*
8. VRSA: vancomycin–resistant *S. aureus*
9. VISA: vancomycin-intermediate *S.aureus*
10. MRSE: methicillin-resistant *S. epidermidis*
11. VRSE: vancomycin-resistant *S. epidermidis*
12. VISE: vancomycin-intermediate *S.epidermidis*
13. VRE: vancomycin-resistant enterococci

14. MIC: minimum inhibitory concentration. Lowest concentration of antimicrobial that will inhibit bacterial growth. A measure of antimicrobial susceptibility.
15. MBC: mean bactericidal concentration.
16 . PCR: polymerase chain reaction. Amplification protocol for specific nucleic acid sequences.
17. SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis. Separation of proteins by size.
18. *femA,B,C*: genes encoding enzymes that are necessary for cell wall cross-linking.
19. *mecA*: gene that encodes penicillin binding protein 2a, a cell wall biosynthesis enzyme.
20. PBP: penicillin-binding proteins. Found in the cell wall and function as enzymes for synthesis of the cell wall.
21. *van A,B,C*: genes that encode a variety of enzymes necessary for vancomycin resistance in *Enterococcus.*
22. Carboxypeptidases: enzymes that break peptide bonds at the carboxy terminus
23. Transpeptidases enzymes that form peptide bonds between amino acids
24. Endopeptidases: enzymes that reversibly cleave peptide bonds between amino acids
25. REL: Readily extractable lipids: lipid fraction extractable by chloroform/methanol (2/1).

CHAPTER ONE

INTRODUCTION

I A Background

A.1. Emergence of Antimicrobial Resistance

As the twentieth century has drawn to a close, the medical community has recently begun to express great concerns regarding the emergence of diseases that had previously not been characterized. Emerging diseases are now redefined as "new, reemerging or drug resistant infections whose incidence in humans has increased within the past two decades or threatens to increase in the near future." (40). A heightened awareness that drug resistance is increasing has prompted the medical community to seek solutions immediately. Antimicrobials were introduced in the twentieth century medical community when Alexander Fleming made the serendipitous discovery of the bactericidal properties found in of the *Penicillium* species. Mass production of this wonder drug (penicillin) in the 1940s resulted in the availability of a reliable, consistent cure for such devastating diseases such as puerperal sepsis, pneumonia, wound infections and sexually transmitted diseases. The decrease in morbidity and mortality from bacterial infections appeared to be the great hope of the antimicrobial era. In the 1950's, chloramphenicol, tetracycline and streptomycin were introduced and the possibility of eliminating bacterial infections appeared within reach (3,17). This led to the development of improved antimicrobials with decreased toxicity to the host, increased toxicity to the organism, greater ease of administration and provided clinicians with cures to previously

debilitating and often-fatal bacterial infections.

Antimicrobial therapeutic failures were noted as early as 1944 and that led to the discovery that an enzyme produced by *Staphylococcus aureus* could destroy the effectiveness of penicillin. By the 1970's, outbreaks with multi-drug resistant organisms began to appear (67). Strains of penicillin-resistant *Streptococcus pyogenes* appeared in 1977 as well as methicillin-resistant *S. aureus* (MRSA). Detection of penicillin-resistant strains of *Neiserria gonorrheae* occurred between 1960 and 1970. The emergence of a multi-resistant strain of *Enterococcus faecalis* in 1989 signaled major problems for the medical community (2).

Bacterial resistance to antimicrobials appears to result for a number of reasons including the theory that resistance originated as a mechanism for bacteria to protect themselves from competing organisms. In fact, the protective mechanisms seen in antimicrobial resistant pathogens appear to be similar to the survival mechanisms used by soil dwelling antimicrobial producing organisms (53). The genomes of these microorganisms code for the production of antibacterial proteins to eliminates other soil-dwelling microorganisms, and protect themselves from the bactericidal effects of their own antimicrobial products.

Mechanisms for development of antimicrobial resistance include the ability of the bacterium to:

1. Alter the target site so the antimicrobial is ineffective. Example: vancomycin resistance in *Enterococcus* (70,71).
2. Alter the proteins necessary to transport antimicrobials to the target site. The Antimicrobial is then rapidly expelled from the organism. Example: expulsion of

tetracycline (24).

3. Overproduce key metabolites. Example: sulfonamide resistance (40,69).
4. Prevent the antimicrobial from reaching the target site. Example: binding of penicillin binding proteins to methicillin by MRSA (10).
5. Duplicate, modify or synthesize enzymes that destroy the antimicrobial. Example: β-lactamase (3,53,58).

Although antimicrobial resistance may have originated as a bacterial survival mechanism, the current spread of antimicrobial resistance throughout the world has posed a severe economic and medical dilemma. There are a number of factors that have contributed to overuse of antimicrobials and the resultant spread of antimicrobial resistance, including the selection of resistance genes (40). Resistance genes can become prevalent when antimicrobials are used extensively in agricultural and food (livestock) industries as growth supplements and control against infection. Agriculture depends on antimicrobial aerosolization to protect produce such as fruits from pathogens. The connection to human medicine is clear. Animals and produce for human consumption contribute not only to the pool of antimicrobials ingested by humans, but also to the transmission of resistant organisms especially in cases of improperly prepared food (53).

When antimicrobials are taken inappropriately, elimination of normal susceptible flora allows resistant organisms to reproduce and dominate in the absence of competing normal flora. Inappropriate antimicrobial uses include prescriptions for antimicrobials when clinical symptoms suggest nonbacterial disease or failure to complete a prescribed dosage of antimicrobial (3,40).

The greatest selective pressure of all, however, appears to be the excessive

amount of antimicrobials used in nosocomial environments. Contamination from clinical personnel, visitors or fomites can lead to bacterial infections in immunologically compromised patients who are being treated with excessive antimicrobial therapy (69).

A.2. Suggested protocols for limiting antimicrobial resistance

When antimicrobials are administered, serum drug concentrations can be compared to established levels necessary to control bacterial growth. The drug concentration required to inhibit susceptible organisms is referred to as the minimum inhibitory concentration (MIC). The concentration of the drug can be increased to inhibit growth when resistant organisms are part of the infective population. This concentration is referred to as the mutant prevention concentration (MPC). Drug levels above MPC are required when the organism has demonstrated multiple mutations, as seen in multiple-resistant organisms. Increasing the dosage of antimicrobials may contribute to host toxicity and may not be an option in effective antimicrobial treatment (24).

Recommendations to decrease the likelihood of developing antimicrobial resistance include the decrease or elimination of antimicrobials fed to livestock and poultry. This is an unlikely alternative solution due to the economic devastation it would create in the agricultural industry. Another recommendation is to separate the antimicrobials into classes of veterinary and human antimicrobials and avoid administering human antimicrobials to animals. An additional suggestion requires the selection of fewer antimicrobials in hospital settings. Theoretically, when bacteria are removed from antimicrobial pressure, a reversion to susceptibility will occur. Unfortunately, this supposition is difficult to prove without jeopardizing patient care. Educating the public as well as health professionals is a viable option, but will take time

to accomplish. Procedures and recommendations to accomplish this have been made by the Center for Disease Control (CDC) (13,66).

A recent study suggests dual or multiple antimicrobial treatments should be used for severe infections as a means to avoid antimicrobial resistance. The theory purports that multiple and separate mutational events would have to occur for total resistance to be established. Treatment can be initiated with antimicrobials that have different mechanisms of action (24). Synergistic treatments such as these have already been established for such pathogens as vancomycin-resistant *Enterococcus* (VRE), MRSA and multiple- resistant tuberculosis (23,48,51).

I B Statement of the Problem

The emergence of multi-resistant antimicrobial plasmids in enterococci indicates that interspecies transfer of resistance plasmids occurs in *vivo*. It has been previously demonstrated that enterococci can transfer the *van* genes of vancomycin resistance to other organisms *in vitro*, but not *in vivo* (73). Vancomycin resistance has been reported in *S. aureus, S. hemolyticus* and *S. epidermidis*, although plasmid transfer has not been confirmed in those organisms (2,17). The mechanism for vancomycin resistance, in the absence of *van* gene transfer remains unexplained. As vancomycin is a last resort antimicrobial for MRSA, the concern exists that staphylococci will become vancomycin resistant upon continued exposure to this drug. In fact, clinical isolates of MRSA have demonstrated reduced susceptibility to vancomycin during long-term treatment (37,38). Although intermediate resistance to vancomycin can be overcome by increasing the dosage of the drug, this results in significant side effects. This raises the question as to

what is the relationship between increasing the dosage and increasing resistance? *Van* genes analogous to the enterococcal *van* genes have not been detected in MRSA clinical isolates with decreased susceptibility to vancomycin. This suggests that the mechanism of vancomycin-resistance in staphylococci differs from that of enterococcal plasmid mediated resistance.

Previous studies indicate that staphylococci may develop either in *vivo* or *in vitro* resistance to vancomycin, and resistance is accompanied by certain morphological, physiological and possibly genetic alterations (19,31,34).

I C Purpose of the Study

C.1. Hypothesis

Vancomycin-resistant isolates appear either *in vivo* or *in vitro* when staphylococcal species are exposed to vancomycin over extended periods of time. Resistance results from genetic mutations that affect proteins exclusive to cell wall synthesis. These mutations result in alterations in cell wall synthesis, which would change cellular morphology, physiology and biochemistry.

C.2. Rationale

Continued exposure of *Staphylococcus* to vancomycin forces the microorganism to develop alternate mechanisms for completing cell wall synthesis. Genetic or post-translational mutations can result in alterations to the proteins that directly affect cell wall synthesis, such as penicillin binding proteins and other transpeptidases. In the absence of some of the staphylococcal penicillin binding proteins, cell wall synthesis not only

continues in resistant strains, but also actually increases (31). These genetic changes may therefore, result in inactivation or altered function of the affected protein.

The purpose of this study is to determine the mechanisms whereby staphylococci develop vancomycin resistance and to evaluate the resultant metabolic, genetic and morphologic changes.

I D Objectives of the Study

In order to evaluate the changes that occur when staphylococcal species are exposed to vancomycin, the following questions will be addressed:

1. Can the minimum inhibitory concentration of vancomycin for *S. aureus* and *S. epidermidis* clinical isolates increase by continuous exposure to increasing concentrations of vancomycin?
2. How do parent and adapted organisms differ biochemically?
3. What are the morphological changes in the staphylococcal strains exposed to vancomycin over extended periods of time?
4. What are the genetic changes in organisms that gain resistance to vancomycin?
5. What are the possible mechanisms of resistance that occur when staphylococci are exposed to vancomycin?

The answers to these questions will be obtained in the following manner:

1. Development of vancomycin-resistant strains of S. *aureus* and *S. epidermidis* through step-wise adaptation.
2. Evaluation of the vancomycin-resistant strains for alterations in morphological, biochemical and genetic characteristics.

3. Examination of the vancomycin-resistant strains for mutations in the *mecA, femB, pbp4* and *coag* genes.
4. Comparison of the vancomycin–resistant and vancomycin-susceptible strains for alterations in protein synthesis.
5. Comparison of the staphylococcal strains for differences in cell wall structure and cell membrane composition.
6. Determination of the mechanism used by staphylococci to survive exposure to vancomycin.

I E Significance of the Study

Long-term use of vancomycin for MRSA infections has resulted in clinical isolates that have demonstrable reduced susceptibility to vancomycin. There is a growing concern that vancomycin will eventually become ineffective against MRSA and there are few antimicrobials available to replace it. Determining the mechanisms of resistance to vancomycin in staphylococcal species may lead to the development of new treatments for these potentially devastating infections (116).

E.1. Clinical Cases of Vancomycin Intermediate *S. aureus*

Since June 1996 (13), several cases of vancomycin intermediate resistant MRSA (VISA) have been reported. The first case was a four-month-old male in Japan hospitalized for a sternal abscess following surgery. Following the isolation of MRSA from the wound, the patient was treated with a 29-day regimen of vancomycin consisting of 45mg/kg/day. The MRSA infection continued and the organism cultured from the wound demonstrated an intermediate resistance to vancomycin at 8 μg/ml (37,38). In July

1997, a 59-year-old male in Michigan developed MRSA after peritoneal dialysis. He was treated with multiple courses of vancomycin after which the MRSA developed intermediate resistance to vancomycin. The patient was eventually treated with trimethoprim and sulfamethoxazole (98). The third case of VISA occurred in New Jersey in a 66 year old male recovering from VRE and MRSA infections. He was treated with vancomycin at a dosage of 0.5 grams daily for 6 months as well as gentamycin and rifampin (98). The VISA strain did not possess *vanA, vanB* or *vanC* genes indicating that vancomycin resistance was not due to conjugative transfer from the VRE. In March of 1998, a 79-year-old male in New York was admitted to the hospital with congestive obstructive pulmonary disease and renal failure. The patient developed a blood stream infection with MRSA. He was treated with multiple rounds of vancomycin for a total of 80 grams over a four-month period. Subsequently a VISA strain was isolated when vancomycin treatment failed (84). In April 1998 in France, a two- year old female with leukemia developed MRSA and was treated with vancomycin at 35mg/kg/day. The isolate was also intermediately resistant to vancomycin (80). A case of VISA in a woman with cancer was reported in China (13).

These clinical cases all have the following similarities:

1. Infections due to MRSA were found in all patients and the MRSA had a vancomycin MIC of at least 8µg/ml. Genotyping of these isolates indicated that the VISA strain was derived from the MRSA strain (37,88).
2. Cases were hospital associated and secondary to MRSA infections.
3. All patients had been treated with vancomycin for a minimum of 18 weeks
4. None of the VISA isolates possessed the *van* A, B or C genes (13,27,80)

(37,88).

5. Patients received dosages of up to 0.5 grams per day of vancomycin.

Interestingly, it is difficult to detect vancomycin intermediate resistant organisms by routine laboratory assays such as Kirby-Bauer disk diffusion. Evaluation of the clinical cases cited above demonstrated the MIC for staphylococcal strains was 8 μg/ml by tube dilution but reported as sensitive according to disk diffusion assays (1). Organisms are considered to be sensitive to vancomycin when the minimum inhibitory concentration (MIC) is less than 4 μg/ml; intermediate at 4-8 μg/ml and resistant at greater than 32 μg/ml. Current recommendations include performing a MIC when treatment failure with vancomycin is suspected (11,13).

E.2. Clinical Cases of Vancomycin Intermediate *S. epidermidis* (VISE)

Vancomycin resistance has also appears in coagulase negative staphylococci (3,17). Detection of clinical isolates of *S. epidermidis* containing heterogeneous vancomycin resistant colonies is accomplished by using salt supplemented vancomycin agar plates. While the MIC of vancomycin-resistant isolates may vary from 3-8 μg/ml, it is clear that decreased susceptibility contributes to antimicrobial failures in these patients (102). In addition to other coagulase negative staphylococcal species evaluated, 31 isolates of methicillin-resistant *S. epidermidis* (MRSE) were isolated from bacteremia patients, and 15 of those isolates demonstrated reduced susceptibility to vancomycin. MRSE was isolated from dialysis fluid in a patient with recurrent peritonitis following dialysis and determined to be vancomycin-heteroresistant. The patient had received 30mg/L /kg vancomycin for 30 days prior to isolation of the organism. Vancomycin therapy was discontinued and the dialysis fluid continued to harbor the vancomycin

resistant MRSE for an additional 30 days (90).

CHAPTER TWO

REVIEW OF THE LITERATURE

II A Resistance to Antimicrobials That Inhibit Cell Wall Synthesis in Gram Positive Bacteria

Gram-positive bacteria account for 51 % of all blood stream isolates and one third of all nosocomial infections according to the National Nososcomial Infection Surveillance (13,67). These microorganisms include the methicillin-resistant staphylococcal species, especially *S. aureus* and multi-resistant enterococci.

Enterococcus is an intestinal inhabitant capable of causing diseases such as endocarditis, septicemia and cystitis (63). The most common enterococcal isolate found in hospital settings is *E. faecalis*, a species that accounts for 85-90% of enterococci detected. This species is typically susceptible or intermediately susceptible to penicillin. This species accounts for 85-90% of enterococcal isolates (48). *E. faecium*, although not isolated as frequently, is highly resistant to β-lactam antimicrobials as well as vancomycin, gentamycin and streptomycin. Enterococcal infections may either arise from endogenous flora or from infections that originate by cross transmission by contacting other patients and from contact with hospital personnel (57). In 1989, a strain of *Enterococcus faecalis* was the multi-resistant isolate to most currently available antimicrobials for enterococci. With the emergence of vancomycin resistance, few other

antimicrobials are available for eradicating this potentially fatal infection. Some synergistic combinations have been used with variable success such as aminoglycosides combinations with β-lactams, aminoglycosides or vancomycin (23,35,48).

II B Development of Resistance to Vancomycin in Enterococci

B.1. Overview of vancomycin mechanism of action

Vancomycin, a glycopeptide antimicrobial, inhibits cell wall synthesis by binding to the terminal D-alanyl-D-alanine dipeptide in the pentapeptide of peptidoglycan. Vancomycin not only inhibits transglycosylation of the peptidoglycan monosaccharides, N-acetyl muramic acid and N-Acetyl glutamic acid, but also transpeptidation (2,21). From all appearances, the peptide fragment of vancomycin binds noncovalently to the D-ala terminus while the carbohydrate moiety sterically blocks the transglycosylation. While it is uncertain whether the binding of vancomycin to the pentapeptide monomer occurs in the membrane or just outside where glycosylation is occurring, vancomycin competes with the cell wall penicillin binding proteins for binding to peptidoglycan and completing cell wall synthesis (46). Vancomycin continues to be a last resort drug used when β-lactam drugs are ineffective, such as in MRSA. Vancomycin is also used in dental prophylaxis, treatment for infections due to *Clostridium difficile* and for enterococcal endocarditis when combined with aminoglycosides. Administered intravenously, except in treatment of pseudomembranous colitis (17,62), therapeutic ranges are from 5 to 40 μg/ml. Microorganisms with vancomycin MICs of less than 4 μg/ml are considered to be susceptible to the drug. Standard dosages are 2 grams per

day, and vancomycin exhibits a post-antimicrobial effective concentration for 2-3 hours (58,98). Renal clearance of vancomycin allows serum levels to approach 30-to 50 μg/ml one-hour post dosage. The recommended serum levels reflect a ten-fold concentration of the vancomycin MIC and these levels are necessary for effective bactericidal activity (84). If serum levels are allowed to increase above the maximum recommended levels, an increase in side effects may result. Side effects can include ototoxicity, nephrotoxicity, fever, chills and phlebitis. Intermediate resistance of an organism to vancomycin is indicated when the MIC is 8-16 μg/ml. Vancomycin MIC of greater than 32 μg/ml indicates total resistance of the microorganism to the antimicrobial. Excessive usage, prolonged treatment and the elimination of susceptible strains (40,43) have facilitated vancomycin resistance.

B.2. Mechanism of resistance in *Enterococcus*

A conjugative plasmid or transposon transfers vancomycin resistance to *Enterococcus*. Although vancomycin has been used since 1958, resistance was first noted in 1989 (2,3). Increased vancomycin usage, particularly throughout the 1980s, has contributed to the development of resistant strains seen presently. Incidences of infection with vancomycin- resistant enterococci (VRE) increased 20 fold from 1989 to 1993.

A total of nine genes contribute to the vancomycin resistance and enterococcal species appear to have accumulated all nine (2). An enterococcal transposon carrying the vancomycin resistant genes can confer one of three different phenotypes, Van A, Van B, and Van C. Van A occurs most commonly and remains the most completely studied (59). Transposon *Tn* 1546 carries five genes that code for the Van A phenotype. Included in this transposon are the *vanS* and *vanR* genes, which encode a two component regulatory

mechanism. The regulatory system directs the transcription of the genes for three structural proteins, Van H, Van A, and Van X. V*anS* senses the presence of vancomycin on the cell wall and *vanR*, signaled by *vanS*, initiates transcription of the other three genes. Van H, a keto acid dehydrogenase enzyme, synthesizes D-lactate from glycerol aldehyde. Van A, a D-alanyl - D-alanine ligase, forms a peptide bond between D-alanine and D-lactate on the pentapeptide. Van X, a carboxypeptidase, removes the terminal D-alanine so that vancomycin does not have a binding site (71).

The resulting action of this enzyme is to synthesize a mucopeptide whose side chain terminates in D-lactate. Vancomycin cannot bind to the D-alanyl-D-lactate dipeptide and without the appropriate binding site, cell wall synthesis continues unconstrained (7,19,21). Recently it has been determined that Van A is greater than 60% homologous to the amino acid sequence of a ligase present in antimicrobial producing organisms such as *Streptomyces toyocaensis*. This raises the interesting possibility that at least some of the antimicrobial plasmids and transposons actually arose from soil dwelling organisms (2,17,21).

II C Staphylococcal Metabolism

C.1. Characteristics of *Staphylococcus:*

Staphylococcus species are gram-positive cocci growing in clusters, catalase positive and capable of growing in the presence of 7.5% NaCl. Species in this genus ferment glucose and are non-motile facultative anaerobes. Further means of identifying *S. aureus* includes assays to determine that the microorganism is coagulase positive,

protein A positive, mannitol fermenter and β- hemolytic. In contrast, *S. epidermidis* is coagulase negative, does not ferment mannitol, is non-hemolytic and does not possess protein A in the cell wall (72,83).

C.2. Peptidoglycan Synthesis in *Staphylococcus*

The amino sugars, N-acetyl glucosamine and N-acetyl muramic acid are synthesized from the fructose-6 –phosphate precursor present in the cytoplasm. Uridine diphosphate (UDP) binds both amino sugars during intermediate synthesis, and then transfers them to undecapronol diposphate (UDPRPP), a lipid carrier. UDPRPP transports the bound amino sugars into the cell membrane. The lipid carrier will escort the amino sugars through the cytoplasmic membrane and periplasmic space to the cell wall. N-acetyl muramic acid also carries a pentapeptide covalently bonded to the lactyl group on carbon #3. The amino acids are added in the cytoplasm by transpeptidases in the following order: L-alanine, D-glutamate, L-lysine, D-alanine, and D-alanine. Once the amino sugars are transported into the cell membrane from the cytoplasm, transglycosylases form glycosidic bonds between the two amino sugars. The resulting disaccharide then crosses across the periplasmic space and into the cell wall, where additional transglycosylases add the disaccharide to the existing cell wall. Penicillin Binding Proteins (PBP), cell membrane bound enzymes, function not only as carboxypeptidases in the removal of the terminal D-alanine, but also as transpeptidases that form a peptide bond between the L-lysine and a pentaglycine bridge (14). The PBPs have high affinity for the β-lactam antimicrobials (thus the name of penicillin binding proteins), and if a PBP is disabled due to β-lactam binding, the other PBPs can function in its place. The *pbp* genes are present on the chromosome and code for the four PBP

proteins of 85, 79,75, and 48 Kilodaltons (KD) respectively (61).

C.3. Mechanism of methicillin resistance in *Staphylococcus*

Methicillin, a β-lactam antimicrobial, binds to the PBPs of gram- positive bacteria and inhibits cell wall synthesis. Methicillin resistance *has* been documented in *S. aureus* as well as coagulase negative *Staphylococcus*. MRSA was first isolated from a clinical source in 1977. This microorganism is the most common cause of surgical wound infections and second only to coagulase negative *Staphylococcus* in bloodstream isolates (83). Symptoms of MRSA infection manifest primarily as bacteremia, endocarditis and pneumonia. Treatments with various combinations of aminoglycosides, rifampin and third generation cephalosporins have been much less effective and inconsistent than treatment with vancomycin (4). The emergence of MRSE as major nosocomial threat is particularly devastating to compromised patients with infections involving indwelling catheters leading to bacteremia (102).

Population studies on the reported clinical cases of VISA and VRSA determined that these cases derived from existing MRSA infections (39,60). VRSA strains do not tolerate the presence of methicillin after long-term exposure to vancomycin has resulted in genetic alterations. Understanding methicillin resistance in staphylococci may assist in developing a hypothesis for vancomycin resistance.

The mechanism of methicillin-resistance has been extensively studied and well documented. MRSA and MRSE strains synthesize the four penicillin-binding proteins present in all staphylococci, as well as a unique PBP named PBP2a. *MecA* gene, 2130 bp in length, codes for the 76kD enzyme. The mecA genes shares homology with the *blaz*

gene, a gene encoding for penicillinase. MecA is located on the staphylococcal genome inserted between the gene *nov*, encoding DNA gyrase, and *spa*, a gene that encodes protein A (41,65). Both MRSA and MRSE possess the *mecA* gene and exposure of either of these species to methicillin induces the translation of *mecA* to PBP2a.

PBP2a has low affinity for β-lactam antimicrobials and fulfills the cell wall synthesis functions when other PBPs are bound and inactivated by the methicillin (15,29). Although *mecA* gene is present on the chromosome, translation of PBP2a does not always occur. When methicillin resistance occurs in the absence of PBP2a, the resistance may be due to additional genes, such as the *blaz* gene (6,77,103).

Other contributing factors to methicillin resistance include auxiliary genes called "factors that enhance methicillin"(*fem*). *Fem* genes flank the *mecA* gene on the staphylococcal chromosome. The *fem* genes codes for transpeptidases that synthesize the pentaglycine cross bridge by adding glycine residues (10,14). Delineation of the functions for the Fem proteins resulted from transposon inactivation studies. The functions are:

Fem A adds glycine #2 and #3 (24), FemB adds glycine #4 and #5 (56), Fem C converts glutamic acid residue to glutamine in the pentapeptide and Fem D is involved in synthesis of peptidoglycan precursors (figure 1). The existence of Fem X has been postulated and its function is to add glycine # 1 to the interpeptide bridge (22). The *femB* gene is not generally present in coagulase negative staphylococci because these species contain serine in the number 4 position of the peptidoglycan cross bridge. However, a *femAB* positive isolate of *S. epidermidis* was identified (100). The remaining Fem proteins are necessary for cell wall biosynthesis as observed through transposon

inactivation studies. Inactivation of *femA* increased autolysis in MRSA isolates that also produced PBP2a (29) whereas inactivation of *femB* increased susceptibility to methicillin and resistance to hydrolases such as lysostaphin (22,52,64). Inactivation of *femC* results in a deficiency of glutamine synthetase, an enzyme that converts glutamic acid to glutamine. In the absence of glutamine synthetase, excess glutamate forms polar bonds with L-lysine. (30,79). The erroneous bonding of D-glutamate and L-lysine results in decreased cross-linking between pentapeptide strands and D-alanyl-D-alanine dipeptides .The alanyl dipeptide can bind the PBP2a, inactivate the PBP and result in a loss of methicillin resistance (40,52,99).

Figure 1 demonstrates the action of *fem* proteins on biosynthesis of the staphylococcal pentaglycine bridge.

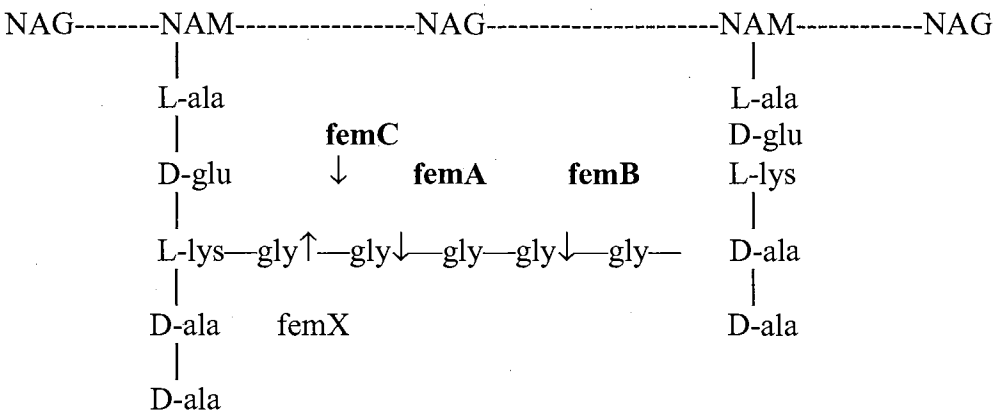


FIGURE 1: Action of *fem* genes on staphylococcal peptidoglycan glycine crossbridge

Methicillin resistance may require up to 12 additional genetic components including *blaz* genes that encode for β-lactamases. In microorganisms that do not possess a *mecA*, methicillin-resistance is still possible through β-lactamase. β-lactamase gene (*blaz)* can be plasmid derived and transferred from microorganism to microorganism through conjugation (10). A methicillin MICs of greater than 8 μg/ml is the criterion for determining if *Staphylococcus* is resistant to methicillin.

EPK I strain of *S. capitis* contains a plasmid, *pACK I* that includes the gene *epr* (endopeptidase resistance). This gene codes for an enzyme that adds serine residues and increases the microorganism's resistance to hydrolases such as lysostaphin and ALE1. The cell walls of S. *aureus* transformed with pACK1 have increased numbers of serine residues in the interpeptide bridge as well as increased resistance to peptidoglycan hydrolases(100). Amino acid sequence of *epr* demonstrates homology to *femAB* gene family, which contributes to the previous methicillin resistance. The presence of *epr* alone, however, does not affect the susceptibility of the organism to methicillin, but may play a complimentary role with *femA* (105).

When the MRSA genome was probed with *femAB*, additional auxiliary genes were located and found to be analogous to the *epr.* These genes, *fmha* and *fmhc* appear to encode for enzymes that also add serine and to confer lysostaphin resistance. The gene *fmhb* may be the hypothesized Fem X, which adds glycine to the number 1 position in the interpeptide bridge (87).

Studies have focused on the *fmt* gene that encodes for the Fmt protein. The exact function of the Fmt protein is currently unknown, but it does not function as a transpeptidase. The Fmt protein shares amino acid homology with carboxypeptidases such as β-lactamase

and PBPs, and this protein is essential for cell wall synthesis (42,105).

The presence of the Fmt protein significantly increases methicillin resistance, however, transposon inactivation experiments revealed that the *fmt* gene is distinct from both the fem and *mecA* genes (47).

II D Vancomycin Resistance in Staphylococci

Prolonged *in vitro* or *in vivo* exposure of staphylococci to vancomycin results in numerous changes that enable the cells to protect themselves against vancomycin. The mechanism of vancomycin resistance in staphylococci remains unknown but may include changes in the bacterial target site as well as changes to the antimicrobial. Staphylococci that have been exposed to vancomycin, *in vivo* and *in vitro,* have been investigated in order to detect alterations to the cell wall, in the cellular proteins and the genome.

D.1. Changes in vancomycin and methicillin susceptibilities among MRSA isolates

VISA clinical isolates have vancomycin MICs of 8 – 16 μg/ml. Studies indicate that VISA originated from MRSA isolates that had reported vancomycin MIC of 1-2 μg/ml and methicillin MIC of >800 μg/ml (39). VRSA, an *in vitro* adaptation of MRSA, achieved vancomycin MIC of as high as 100 μg/ml, whereas the methicillin MIC was 1-2 μg/ml. Prior to adaptation, the MRSA was susceptible to vancomycin at 1-3 μg/ml and resistant to methicillin at greater than 1200 μg/ml. The apparent replacement of antimicrobial resistance profile was a consistent observation in all in vitro adapted strains and might play a role in treatment of adapted organisms (39).

D.2. Examination of the cell wall alterations by electron microscopy

Examination of VISA and VRSA isolates by transmission and scanning electron Microscopy revealed VISA isolates (vancomycin MIC of 8 μg/ml) had thickened cell walls and increased cellular septa (33). VRSA isolates (vancomycin MIC of 100 μg/ml) had thickened cell walls and septa, multicellular aggregates and extracellular material on the surface of the cell (78). When allowed to grown in sublethal amounts of vancomycin, isolates of susceptible S. *aureus* also developed thickened cell walls and abnormal morphology (38). VRSA isolates, vancomycin MIC of 30 μg/ml, had similar morphologies of thickened cell walls, multiple septa and abnormal surface morphology (18,49).

D.3. Compositional changes in the cell wall

Studies comparing hydrolases activity on the cell walls of MRSA and VRSA showed that the VRSA was more resistant to lysostaphin than MRSA (44). Wild-type *S. aureus* transformed with *pACK1* contained a significant decrease in the amount of glycine as well as an increase in the amount of serine as compared to non-transformed *S. aureus*. Transformed *S. aureus* resisted hydrolase degradation whereas non-transformed *S. aureus* did not. When radioisotope labeled N-acetyl glucosamine was added into growing cultures of VRSA and MRSA, the rate of cell wall synthesis increased in the VRSA as compared to MRSA (77). VRSA released labeled N-acetyl glucosamine into the media at a slower rate than MRSA and produced increased amounts of precursor monomers (31). Increased cell wall synthesis and decreased autolysis are consistent with the thickened cell walls seen in VRSA. High-pressure liquid chromatography (HPLC) of the cell walls of VRSA and MRSA showed an increase in glutamate residues in the

VRSA (92). Results suggest that glutamine synthetase was inactivated, the enzyme coded for by the *femC* gene (30,31). HPLC also detected D-alanyl-D-alanine dipeptides in the VRSA cell walls as well as a decrease in the cross linkage of the mucopeptide. These alterations were heterogeneous in this study and it was hypothesized that the dipeptides might provide binding sites for vancomycin 32,80,93).

D.4. Protein alterations in vancomycin-resistant strains

VRSA and VRSE contain alterations in various PBPs, particularly PBP4 and PBP2a. When PBP2a is translated in both of these organisms, studies show that the protein does not contribute to vancomycin resistance (6,57). PBP4, a D-alanine carboxypeptidase, appears to be absent in the VRSA and VRSE strains (89,90). PBP4 removes the terminal D-alanine of the mucopeptide and facilitates cross-linking (89). Deletion of β-lactamase and β-hemolysins has also been demonstated in vancomycin-resistant staphylococci (81). Although these proteins may be synthesized, it is possible they are not secreted because of the thickened cell wall (68). Coagulase activity in VRSA is either missing or prolonged (77,107). Proteins present within the cell wall, such as protein A and catalase are still expressed (81).

A 39kD protein analogous to the *Enterococcus* NAD+dependent D-Lactate Dehydrogenase is not only present, but also overproduced in VRSA clinical isolates. The gene for this enzyme is found in the VRE plasmid, *vanH,* and the enzyme functions to remove the terminal D-alanine and replace it with D-lactate. This protein has been discovered in the cytoplasm of VRSA but it does not function in the same manner as in enterococci. No D-lactate precursors have been located in VRSA and inhibition studies demonstrated that the enzyme does not contribute to the mechanism of vancomycin

resistance (59,64).

D.5. Genetic changes

The S. *aureus* genome is a 2.7 mbp single circular chromosome that includes the genes for *mecA* (2.1kbp), *fem*, *pbp* and other regulatory genes associated with methicillin resistance (65). Inactivation of any of the f*em* genes, regardless of the presence of *mecA,* results in decreased resistance to methicillin and possibly the increased resistance to vancomycin. Transposon inactivation studies of the VISA and VRSA isolates using *fem* genes concluded that the inactivation of *femB* resulted in the substitution of serine for a glycine residue in the pentapeptide, increased resistance to endopeptidases, but not to increase vancomycin resistance. Inactivation of *femC* did not alone increase the resistance to vancomycin, but it may contribute when other mutations occur (102,108).

Transposon inactivation of the *mecA* gene in VRSA resulted in decreased resistance to methicillin, but not to increased resistance to vancomycin (19). Inactivation of *pbp4* gene has been reported in VRSA and the deletion of the PBP4 protein resulted with increased D-alanyl-D-alanine dipeptide and decreased cross-linkage of the pentapeptide (89).

The *ddh* gene is overexpressed in VRSA and inactivation of the gene by transposon insertion only slightly increases the resistance of *S. aureus* to vancomycin (8). No terminal D-lactate in the mucopeptide of vancomycin resistant staphylococci has yet been discovered, so the function of the dehydrogenase in *Staphylococcus* remains to be elucidated.

D.6. Summary of the changes in vancomycin resistant staphylococci

Studies comparing MRSA and VRSA have shown:

1. The cell walls of VRSA are thicker and have abnormal morphology.
2. Cell wall synthesis is increased and autolysis is decreased in VRSA. Cell walls appear to have D-alanyl-d-alanine dipeptide termini capable of binding vancomycin.
3. VRSA cell walls are more resistant to lysostaphin.
4. Secretion of free coagulase, β-lactamase and β-hemolysins decrease as vancomycin resistance increases in VRSA.
5. Inactivation of *mecA*, femB or *femC* genes alone does not increase resistance to vancomycin.
6. The presence of NAD+-Lactate Dehydrogenase does not produce D-alanyl-D-lactate dipeptides in the pentapeptide and does not increase vancomycin resistance.
7. Methicillin resistance decreases as vancomycin resistance increases.
8. Presence or absence of PBP2a in VRSA does not increase resistance to vancomycin.
9. *Pbp4* is absent in VRSA but present in MRSA.
10. The presence of *ddh* only slightly increases resistance to vancomycin.

CHAPTER THREE

METHODOLOGY

III A Bacterial Strains and Culture Conditions

The Microbiology Laboratory of St.John's Hospital (Tulsa, OK) generously provided the MRSA clinical isolate. This isolate did not ferment mannitol, but it was positive for coagulase and protein A. Upon subsequent reculturing of this isolate, it was discovered that the original culture included a mixture of MRSA and MRSE. Confirmation of the staphylococcal species was made by 16s rRNA analysis (20). MRSE adapted to vancomycin resistance but MRSA was unable to survive the adaptation process. The DNA isolated from MRSA was used as a control in the DNA analysis.

Carolina Supply (Burlington, NC) is the source for S. *aureus* ATCC 12598 (methicillin-susceptible), designated as wild-type *S. aureus* (WTSA).

Wild-type *S. epidermidis* (WTSE) was a laboratory strain used in the teaching program at OSU-COM (Tulsa, OK).

Muskogee Veteran's Hospital (Muskogee, Oklahoma) generously supplied a clinical isolate of methicillin susceptible, β-lactamase positive *S. aureus*, designated as strain 7695. These strains were identified by typical laboratory methods as well as by 16S rRNA analysis (20) (Midilabs, Newark, DE). Minimum inhibitory concentrations for vancomycin and methicillin were measured as described below.

Muskogee Veteran's Hospital was also the source for a clinical isolate of vancomycin-resistant *Enterococcus faecalis* (VRE). This isolate was used as a control for the vancomycin susceptibility tests as well as vancomycin screening plates.

III B Exposure of Staphylococcal Species to Vancomycin

B.1. Step-wise adaptation

Strain 7695 and MRSE adapted to vancomycin-resistance by the procedure of Conrad, et.al. (16). Using the following protocol, isolated colonies from each strain were placed into individual flasks containing 50 ml of Mueller–Hinton broth supplemented with Mg2+ (12.5 mg/L) and Ca2+ (25 mg/L)(CSMH). Flasks were incubated at 37°C and aerated with vigorous shaking until the optical density of each culture was greater than 1.0. Optical density readings of greater than 1.0 at 620nm are considered to be maximal growth. An aliquot from each flask was placed into separate side arm flasks containing 50 ml of CSMH broth and 1x MIC vancomycin.

Each flask reached an optical density of greater than 1.0 at 620 nm after 24-48 h at 37°C. Identities of the isolates were continually confirmed by gram stains and mannitol salts agar plates. An aliquot from each flask was placed into 50 ml of fresh CSMH broth containing 2xMIC vancomycin. The process of evaluation, isolation and incubation of each isolate continued in CSMH with increasing concentrations of vancomycin at two fold concentrations. Vancomycin concentrations were increased to 32 μg/ml when the cultures grew consistently at vancomycin concentrations > 20 μg/ml. Each culture containing 32 μg/ml vancomycin reached maximal growth at 72 h at 37°C.

WTSA and WTSE were used as control cultures and both reached maximal growth at 24 h. Working cultures of all strains were routinely maintained on blood agar plates.

B.2. Assessment of adapted strains

The preparation of antimicrobial selective agar involved adding filter-sterilized antimicrobial to autoclaved melted agar media that was maintained at 45°C. Selective agar was poured into Petri plates until the bottom of the plate was covered. After allowing the agar to harden, the plates were examined for accuracy by inoculating the vancomycin plates with clinical isolates of VRE and the oxacillin plates with MRSE. Culture fluids obtained during the adaptation process were inoculated onto Mueller-Hinton agar plates containing either 10μg/ml vancomycin or 8 μg/ml oxacillin.

B.3. Reversion Experiment

Vancomycin-resistant strains were transferred onto antimicrobial-free blood agar plates daily for a total of 20 days. After 20 days, the resistant strains were evaluated by inoculating the colonies from day 1 and day 20 onto vancomycin containing agar plates and MIC assays. In parallel experiments, the adapted strains were also transferred into antimicrobial free CSMH broth for 6-8 passages. Results from the assays performed at day 20 were compared to assay results of the cultures maintained in the presence of vancomycin.

III C Antimicrobial Susceptibility of *Staphylococcus*

Standard protocols and manufacturer's recommendations determined antimicrobial susceptibilities. These techniques included minimum inhibitory

concentration (MIC) by microtiter plates, disk diffusion, and the Etest method (5,72). All antimicrobials were obtained from Sigma (St. Louis, MO). Stock solutions of antimicrobials were filter-sterilized and frozen at -20°C until used. Overnight broth cultures were adjusted to an optical density of 0.20 at 620 nm. The cultures were either swabbed onto CSMH agar plates prior to adding antimicrobial disks or strips, or dispensed in 5 µl aliquots into microtiter wells containing appropriate dilutions of the antimicrobials.

For the microtiter dilution, an overnight culture was adjusted to an optical density of 0.2 at 620nm prior to pipetting 5 µl of the diluted culture into wells containing dilutions of appropriate antimicrobial. The optical density of the microdilution wells was measured after incubation for 24 hours at 37°C. The lowest dilution of antimicrobial that inhibited growth was recorded as the end point. Assays were performed in triplicate. Disk diffusion and Etest were performed according to manufacturer's recommendation (5).

Overnight cultures were diluted to an optical density of 0.2 at 620 nm, and then swabbed evenly onto the surface of Mueller-Hinton agar plates. Either antimicrobial disks or Etest strips were placed onto duplicate inoculated plates. After the agar plates had incubated overnight at 37°C, the zone of inhibition for each disk was measured or the concentration of inhibition read directly from the Etest strips. All assays included positive control cultures of VRE, MRSE, WTSA and WTSE.

Minimum Bactericidal Concentration (MBC) was determined by placing 100µl of culture broth from each microtiter well onto CSMH agar plates. The antimicrobial dilution that killed 100% of the bacteria was recorded as the end point (72).

III D Biochemical Assays

D.1. Proteins

Assays for coagulase, protein A, β-lactamase and catalase were performed as recommended by NCCLS guideline (72). WTSA and WTSE were included as controls for each assay. For the coagulase test, colonies from an overnight blood agar plate were added to 0.5ml rabbit serum (Sigma, St. Louis, MO) and the serum tubes were incubated at 37°C for 4 h. Clotted serum indicated a positive result. Negative results were allowed to incubate overnight at 37°C and the results were recorded as negative if the serum had not clotted within 24 hours.

Protein A is a cell wall surface protein unique in *S. aureus*, which binds to the Fc portion of IgG. Colonies from overnight cultures are mixed with latex beads labeled with IgG and observed for agglutination (Staphyloslide®, BBL; Cockeysville, MD). If agglutination occurred, isolates were considered to be positive for the protein (25).

Nitrocefin disks were used to determine the presence of β- lactamase that identify the enzyme by a colorimetric assay (Remel, Lenexa, KS). Overnight colonies are added to a hydrated nitrocefin disk and allowed to react for 5-30 min. at room temperature. The test is positive when the disk turns yellow (72).

Catalase is an enzyme that converts 30% hydrogen peroxide to O_2 and H_2O. A drop of 30% hydrogen peroxide, placed onto colonies from an overnight culture, will bubble if the strain is positive for catalase.

D.2. Carbohydrate fermentation

Carbohydrate fermentation profiles were performed by the method of API Staph™. (BioMerieux, Hazelwood, MO). Colonies from overnight blood agar plates were placed in the diluent vial and dispensed into plastic strips containing the individual carbohydrates. Inoculated strips were placed into the kit chamber and incubated at 37°C overnight. Results were interpreted at 24 h as suggested by the manufacturer's instructions. Results for each isolate was compared with the master list supplied with each kit (45).

III E Growth, Killing Curves and Lysostaphin Associated Degradation

E.1. Growth and generation time

All strains were inoculated onto blood agar plates and allowed to incubate overnight at 37°C. Four to six colonies were picked from each plate and inoculated into 50 ml. CSMH side arm flasks. Flasks were incubated at 37°C with aeration. Each culture grew until absorbance was at least 0.1 at 620nm. Absorbance was measured and recorded at 30-min intervals for a total of nine hours.

Prior to incubating the flasks, 100µl from of each culture flask was inoculated onto CSMH agar plates. The agar plates were incubated at 37°C overnight and the colony count was determined. At the end of the 9 h incubation period, 100µl of each flask was placed into 900 µl CSMH broth (1:10). An aliquot of 100 µl from the 1:10 dilution was placed into another tube of 900 µl of broth (1:100) and the ten fold dilutions were repeated for a total of five tubes, with the final concentration of 10^5. A sample of 10 µl

from each dilution was inoculated onto CSMH agar plates and spread to separate colonies. After an overnight incubation at 37°C, the colonies on each plate were counted.

Calculations of doubling time were based on the following formula: Generation time $=(t-t_0)$ /logN-logN_0/. 301 (12). g=generation time; logN= number of bacteria at 9 hours and logN_0= number of bacteria initially; t- t_0= length of time in culture (12).

E.2. Killing Curve

Ten-milliliter aliquots of each isolate were placed into 50 ml CSMH flasks containing 32 μg/ml vancomycin. Initial absorbance of each culture was adjusted to 0.1 at 620 nm prior to the addition of vancomycin. After vancomycin was added, the flasks were incubated at 37°C with aeration and the absorbance was measured every 30 min. Optical density of each flask continued to be measured for a total of 9 h.

E.3. Lysostaphin Resistance

WTSA, MRSE, strain 7695, VRSA, WTSE and VRSE were grown overnight at 37°C in 50ml flasks containing vancomycin-free CSMH. The next day the absorbance at 620 nm was adjusted to 0.6 for all cultures. Each flask received lysostaphin at a concentration of 1unit/ml (Sigma, St. Louis, MO) and incubation continued at 37°C. Absorbance at 620nm was recorded every ten minutes for a total incubation time of one hour.

III F Electron Microscopy

Each isolate was grown in two separate flasks of 500 ml of vancomycin-free CSMH. The cultures were incubated for 4 h at 37°C. A solution of 10mM Tris-HCl (pH

8.0) buffer containing 32 μg/ml of vancomycin was added to one flask of each isolate. The other flask received a solution of 10mM Tris-HCl buffer (pH 8.0) containing no vancomycin and both flasks were incubated at 37°C for additional 6 h. Cells from each culture were harvested for transmission and scanning electron microscopy by centrifuging the culture fluids at 10,000x *g* for 15 min. The cell pellets were washed three times in 10mM Tris-HCl, (pH 8.0) to remove the CSMH and vancomycin. Washed and pelleted bacterial cells were fixed in 1.6% glutaraldehyde and sodium cacodylate buffer and then refrigerated overnight at 4°C. Samples were dehydrated through graded ethanol series and then placed in increasing amounts of resin/acetone. Samples were embedded overnight at 64°C in fresh resin (9).

F.1. Scanning Electron Microscopy

Samples for scanning electron microscopy were fixed in osmium tetraoxide, rinsed with cacodylate buffer and refrigerated overnight at 4°C. Samples were fixed onto poly-lysine coated coverslips, dehydrated by ethanol series and dried. Coverslips were mounted onto aluminum stubs, coated with gold/palladium and viewed by electron microscope (9).

F.2. Transmission Electron Microscopy

Samples for transmission electron microscopy were cut with a microtome into .5μm sections, placed onto copper posts and stained with Mallory's stain. Specimens were post-stained with uranyl acetate and Reynold's lead stain and examined by electron microscopy by standard procedures (9). Measurements of the cell walls were made by comparing the diameter of the cell with and without the cell wall thickness in at least 3 separate locations per cell.

III G Evaluation of Membrane Bound Proteins

G.1. Extraction of cell membrane proteins

Strains were grown in flask cultures containing either one-liter of vancomycin-free CSMH broth or one liter CSMH broth and 32 μg/ml vancomycin. After maximal growth was achieved, the culture fluids were centrifuged at 10,000x *g* for 15 min. Pelleted cells were washed three times with 10mM Tris-HCl, (pH 8.0) and then suspended in lysis buffer containing one unit/ml of lysostaphin and ten units/milliliter of lysozyme (Sigma, St. Louis, MO) in 10mM Tris-HCl, (pH 8.0). Treated cells incubated overnight at 37°C with gentle stirring. Cell cultures were poured into 50 ml conical plastic tubes and lysed cells were sonicated three times for 30 s bursts. Sonicated cells centrifuged at 14,000x *g* for 30 min to pellet cellular debris. The supernatant was removed to ultracentrifuge tubes before centrifugation at 170,000x *g* for one hour to pellet cell membrane proteins. Protein pellets were collected by washing the pellet off the side of the centrifuge tube with 10mM Tris-HCl, (pH 8.0) (19).

Protein concentrations were determined by the bicinchoninic acid assay (BCA). A standard curve of bovine albumin was prepared from a stock solution of 1 mg/ml bovine albumin. BCA reagent was added to 50μl of each standard or membrane protein preparation, tubes were mixed and allowed to incubate at 25°C for one hour. Tubes were transferred into spectrophotometer cuvettes and optical density was measured at 563nm against a water blank. Membrane protein concentrations were determined from the standard curve (99).

The protein concentration of the membrane preparations was adjusted to 2.0 mg/ml by diluting the sample with 10mM Tris-HCl, (pH 8.0). Cell membrane proteins were frozen at -20°C until needed for further assay.

G.2. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE gels were formed according to the standard Laemmli procedure (50). Polyacrylamide gels were prepared by using 10% separating gel and 4% stacking gel. Both 6 cm x 8 cm and 16 cm x 20 cm gels were assembled. Membrane protein samples were thawed, mixed in equal volume with sample buffer containing glycerol and β-mercaptoethanol and heated for 5 min. at 95°C. Protein concentrations were adjusted to 200 μg/ml and 25 micrograms of protein was added to each lane (75). A molecular weight standard of 10kD increments was placed into the first lane. The smaller gels were run for 45 minutes at 100 ma and the larger gels were run for 4.5 hours at 30 ma. Gels were placed in Comassie blue stain for 30 min to one hour, and then destained in glacial acetic acid-methanol (3/1). Destained gels were photographed for a permanent record. The molecular weights of the cell membrane proteins were calculated by the following measurements: distance traveled in cm by protein band/distance in cm for total length of the gel x distance traveled in cm of molecular weight standard closest to the unknown protein band (50).

G.3. Native Gel Electrophoresis

Native gel electrophoresis was performed according to the standard Laemmli procedure (50). The cell membrane proteins were incubated for 30 min. at 37°C with fluorochrome-labeled penicillin (Bocillin, Biorad, Hercules,CA), in a 3/1 ratio of protein to labeled penicillin. Labeled proteins were mixed with equal volumes of sample buffer

containing glycerol but without β-mercaptoethanol. Samples were heated at 95°C for 5 min and then added to the wells of the stacking gel at a concentration of 50 micrograms. Gels electrophoresed for 45 minutes at 100 ma.

Gels were rinsed in distilled water for 5-10 min, then evaluated and photographed over UV light (115).

III H DNA Analysis

H.1. Extraction of genomic DNA

Genomic DNA was isolated by phenol/chloroform extraction as adapted from the procedure of Flamm (26). Pelleted and washed cells were first lysed with lysostaphin as described in the procedure for membrane protein extraction. Lysed cells were mixed with an equal volume of a phenol/chloroform/isoamyl alcohol (25/24/1) solution. Tubes were slowly rotated to prevent shearing of genomic DNA, and then centrifuged at 10,000x *g* for 5 min to pellet precipitated proteins. The aqueous layer was removed and mixed with equal volumes of chloroform to remove contaminating proteins. After centrifugation at 10,000x *g*, the aqueous layer was removed and added to two volumes of ice-cold absolute ethanol and 0.1 volume of 3M sodium acetate. The extraction tubes were gently rotated and placed in an ice bath to facilitate the DNA precipitation. When the solution became viscous, the DNA was spooled onto a glass rod and placed into a tube containing 80% ethanol. The tube was gently rotated to remove any contaminating proteins from the DNA, and then the tube was centrifuged at 10,000x *g* for 5 min. The ethanol was removed completely from the DNA pellet and the DNA was allowed to air

dry at room temperature. The dried DNA was reconstituted in Tris-EDTA (pH 8.8) buffer and maintained at 4°C until needed. DNA concentrations were calculated by measuring the absorbance at 280nm.

H.2. Polymerase Chain Reaction

Genomic DNA was placed into separate PCR reaction tubes at a concentration of 0.1 μg/μl. The following reagents were added to each tube:

1. 10x buffer (100mM Tris-HCl) (pH 8.3)
2. 11mM MgCl
3. 100 μM dNTPs
4. purified water
5. 1 unit/μl Taq polymerase

Primer pairs were synthesized by the core facility at Oklahoma State University (Stillwater, OK). The lyophilized primers were diluted to working concentrations of 20μM per primer. Total volume for each PCR reaction was 50 μl per tube.

Table I provides a list of the primer pairs used in these experiments (6,54,55,76,104,110).

TABLE I : Primers used in this study:	
MecA (651BP)	5'CTCAGGTACTGCTATCCAC 3'FP
	5' CACTTGGTATATCTTCAC 3' RP
*Coag (*131BP)	5' GCTTCTCAATATGGTCCGA 3'FP
	5' GCTTCTCAATATGGTCCGA 3'RP
PBP4 (2076BP)	5' ATAAGACCCACTGGCCATGATAG 3'FP
	5' CTGGGGACAAAAGAAGACGATG 3' RP
FemB (449 BP)	5' TTACAGAGTTAACTGTTACC 3'FP
	5'ATACAAATCCAGCACGCTCT 3'RP
(*BlaZ*) (2000BP)	5'ACTCTTTGGCATGTGAACTG3'FP
	5'CATAACATCCCATTCAGCCA3'RP

Table 1: Summary of PCR Primers Used in Experiments

Reaction tubes were placed in a thermocycler (Genemate Genius™) and reaction temperatures were set according to standard protocols (6, 54, 55,76, 104, 110). Completed reactions were kept at 4°C until assayed by agarose gel electrophoresis. Agarose gels were prepared by mixing 0.8% or 1.6% agarose diluted with Tris-Edta buffer. Ethidium bromide was added to the agarose solution prior to pouring the gel into a preformed mold. Loading dye (5x) was added to each PCR reaction mixture then 15µl of each reaction fluid was placed into a well. Gels were electrophoresed for 1 to 1.5 h at 90 volts, viewed over UV light and evaluated for products of expected base pair length.

Photographs of the gels were taken with both digital and Polaroid cameras.

III I Cellular Components Assays

Cultures of strain 7695 and VRSA were placed into flasks containing vancomycin-free CSMH and incubated overnight at 37°C. A separate culture of VRSA was placed into a flask containing vancomycin was also incubated overnight at 37°C. After overnight growth, culture fluids were centrifuged at 14,000x *g* for 15 min to pellet the cells. The supernatant was removed and the pelleted cells were washed twice in 10mM Tris-HCl (pH 8.0). The remaining fluid was removed completely from the cell pellet before the tube was weighed.

I.2. Readily Extractable Lipids

Each cell preparation was hydrolyzed with 5% NaOH in 50% methanol for 30 min at 100°C. Methyl esters of the fatty acids were formed with Boron Trichloride-methanol and extracted with chloroform/hexane. Esters of the fatty acids were dried under gentle nitrogen stream and assayed by standard procedure for gas/liquid chromatography procedures (98). Calculations of the fatty acid esters were evaluated from peaks detected after 1.5 minutes of chromatography elution to 29 minutes of elution time. That time period accounted for the elution of 100% of fatty acid esters extracted.

CHAPTER FOUR

RESULTS

IV A Step-wise Adaptation of Staphylococci

WTSA, strain 7695, MRSE and WTSE, grown in the presence of 3μg/ml vancomycin, reached maximal growth at 24 h. When the isolates were placed into flasks containing CSMH and 6 μg/ml of vancomycin, maximal growth was reached at 24 h. When the growth medium contained 12μg/ml of vancomycin, MRSE and strain 7695 reached maximum growth at 48 h. WTSA and WTSE did not grow at this concentration of vancomycin. In media containing 20μg/ml vancomycin, MRSE and strain 7695 cultures achieved maximum growth at 72 h. MRSE and strain 7695 cultures were repeatedly cultured in CSMH containing 20 μg/ml of vancomycin until both cultures consistently achieved maximum growth at 48 h. Gram stains were done on each isolate and all strains continued to appear as gram-positive cocci with no contaminating gram-negative bacteria present.

Concentration of vancomycin was increased to 24μg/ml with maximum growth rate occurring at 48 h and finally the concentration of vancomycin for MRSE and strain 7695 increased to 32 μg/ml. The cultures achieved stationary phase at 48 h and were maintained in CSMH containing 32 μg/ml vancomycin. The adapted strains were designated as VRSE (adapted from MRSE) and VRSA (adapted from strain 7695).

Culture fluids from VRSE and VRSA were inoculated onto agar plates containing

either vancomycin (32 μg/ml), oxacillin (8μg/ml) or both antimicrobials. Both VRSE and VRSA grew on the vancomycin agar plate, but neither grew on the other plates. MRSE and strain 7695 isolates grew on oxacillin agar plates, but not on vancomycin or combined agar plates. After seventeen daily passages onto antimicrobial free blood agar plates, VRSE and VRSA isolates grew on vancomycin-containing agar plates, but not on oxacillin agar plates. None of the isolates grew on the combination vancomycin/oxacillin agar plates.

VRSE did not ferment mannitol; consistent with an observation noted with the original MRSA culture. MRSE, VRSE, strain 7695 and VRSA were evaluated for 16s rRNA (20). Strain 7695 and VRSA were confirmed to be *S. aureus* strains, while MRSE and VRSE were confirmed to be *S. epidermidis.* The MRSE colonies remained viable in the presence of 32 μg/ml of vancomycin, whereas, the MRSA did not.

IV B Antimicrobial Susceptibility

B.1. *Staphylococcus aureus*

Strain 7695 was initially susceptible to vancomycin concentrations greater than 2 μg/ml, but resistant to both methicillin and penicillin G at concentrations greater than 256 μg/ml. During the step-wise adaptation, the strain 7695 was able to grow in the presence of increasing amounts of vancomycin, but the vancomycin MIC was a dilution lower. This anomaly continued until VRSA grew consistently in 32 μg/ml. Minimal Bactericidal Killing (MBC) of vancomycin was 128μg/ml. VRSA strain was inhibited by both methicillin and penicillin G a concentration of 1 μg/ml. MIC assays performed after isolates were passed on antibiotic-free agar plates confirmed that the strains retained

vancomycin-resistance but did not revert to methicillin-resistance during this process.

B.2. *Staphylococcus epidermidis*

MICs assayed by both Etest and microdilution methods established that the MRSE was susceptible to vancomycin at 2 μg/ml and resistant to both methicillin and penicillin G at >256 μg/ml. Step-wise adaptation proceeded, but the MIC during adaptation often measured one dilution lower than the concentration of vancomycin in which the organism was growing. When VRSE grew readily in the presence of 32 μg/ml of vancomycin, the MIC was also 32 μg/ml. Both penicillin G and methicillin inhibited VRSE at a concentration of 1μg/ml.

TABLE II **SUMMARY OF ANTIMICROBIAL SUSCEPTIBILITIES IN STAPHYLOCOCCAL STRAINS**

		STAPHYL0COCCAL STRAINS					
Test Procedure	**WTSA**	**MRSA**	**7695**	**VRSA**	**WTSE**	**VRSE**
Disk Diffusion							
Ampicillin	S	R	R	S	S	S
Oxacillin	S	R	S	S	S	S
Penicillin G	S	R	R	S	S	S
Vancomycin	S	S	S	int	S	R
Linezolid	S	S	S	S	S	S
Synercid	S	S	S	S	S	S
Microdilution							
Penicillin G	1	256	**256**	**1**	1	1
Oxacillin	1	256	1	1	1	1
Vancomycin						
MIC	1	2	2	**32**	2	**32**
MBC	2	4	4	**128**	2	**128**

Table II: Summary of antimicrobial susceptibilities of staphylococcal strains to selected antimicrobials. MIC and MBC are measured in μg/ml.

IV C Biochemical Assays

C.1. Proteins

Strains of MRSA and VRSA were positive for coagulase and protein A. However, VRSA required 24 h of incubation at 37°C in order to produce a positive coagulase result. Strain 7695 was positive for β-lactamase, but VRSA was negative for β-lactamase.

WTSE and VRSE were negative for coagulase and proteinA as expected (72). Both strains of *S. epidermidis* were negative for β-lactamase.

C.2. Carbohydrates

All strains were assayed for carbohydrate fermentation as described previously. The original culture of MRSA did not ferment mannitol and continued to be a mannitol nonfermenter. Fermentation profiles of all other strains were consistent with the expected results of the species (45).

Table III is a summary of the biochemical assay results for all staphylococcal isolates.

TABLE III **Summary of Biochemical Assays**

ASSAY PROTEIN ASSAY	WTSA	MRSA	7695	VRSA	WTSE	VRSE
Protein A	POS	POS	POS	POS	NEG	NEG
B-lactamase	NEG	NEG	POS	**NEG**	NEG	NEG
Coagulase	POS*	POS*	POS*	**POS****	NEG	NEG
Catalase	POS	POS	POS	POS	POS	POS
CARBOHYDRATE FERMENTATION						
Glucose	POS	POS	POS	POS	POS	POS
Fructose	POS	POS	POS	POS	POS	POS
Sucrose	POS	POS	POS	POS	POS	POS
Lactose	POS	POS	POS	POS	POS	POS
Mannitol	POS	**NEG**	POS	POS	NEG	NEG
*pos@4hr **pos@24						

Table III Summary of Biochemical Assays for Staphylococcal species

IV D Growth Curve

D.1. *S. aureus* strains:

Strain 7695 began logarithmic growth at 2 h of incubation at 37°C and continued until maximal growth was reached at nine hours when the optical density reached 1.0. Strain 7695 did not increase in turbidity during a nine hour period when cultured in the presence of 32 μg/ml of vancomycin. The bactericidal effects of vancomycin were confirmed by the lack of growth when strain 7695 was inoculated onto MH agar plates overnight at 37 °C.

VRSA achieved logarithmic growth at 4 h and growth continued for a total of 12

hours, at which time, the optical density of the culture reached 0.52 at 620nm. VRSA did not begin logarithmic growth for almost 8 hrs when grown in the presence of 32 μg/ml vancomycin but growth continued for 24 h (Figure 2).

Generation times were calculated for each strain as follows: strain 7695: g= .301(9-2)/log 7-log3=31.6 minutes. VRSA: g= .301(12-4)/log6-log2.84)=45 minutes.

D.2. *S. epidermidis* strains:

MRSE achieved logarithmic growth at 3 h and reached maximal growth at 13 h. The MRSE strain did not grow when cultured in the presence of vancomycin (32μg/ml). The VRSE strain began logarithmic growth at 30 hours of incubation at 37°C and reached maximal growth continued at 48 h. VRSE was not grown in the presence of vancomycin for this experiment (Figure 3).

Generation times for the *S. epidermidis* strains were MRSE g= 23 min and VRSE g= 46 min.

IV E Lysostaphin Resistance

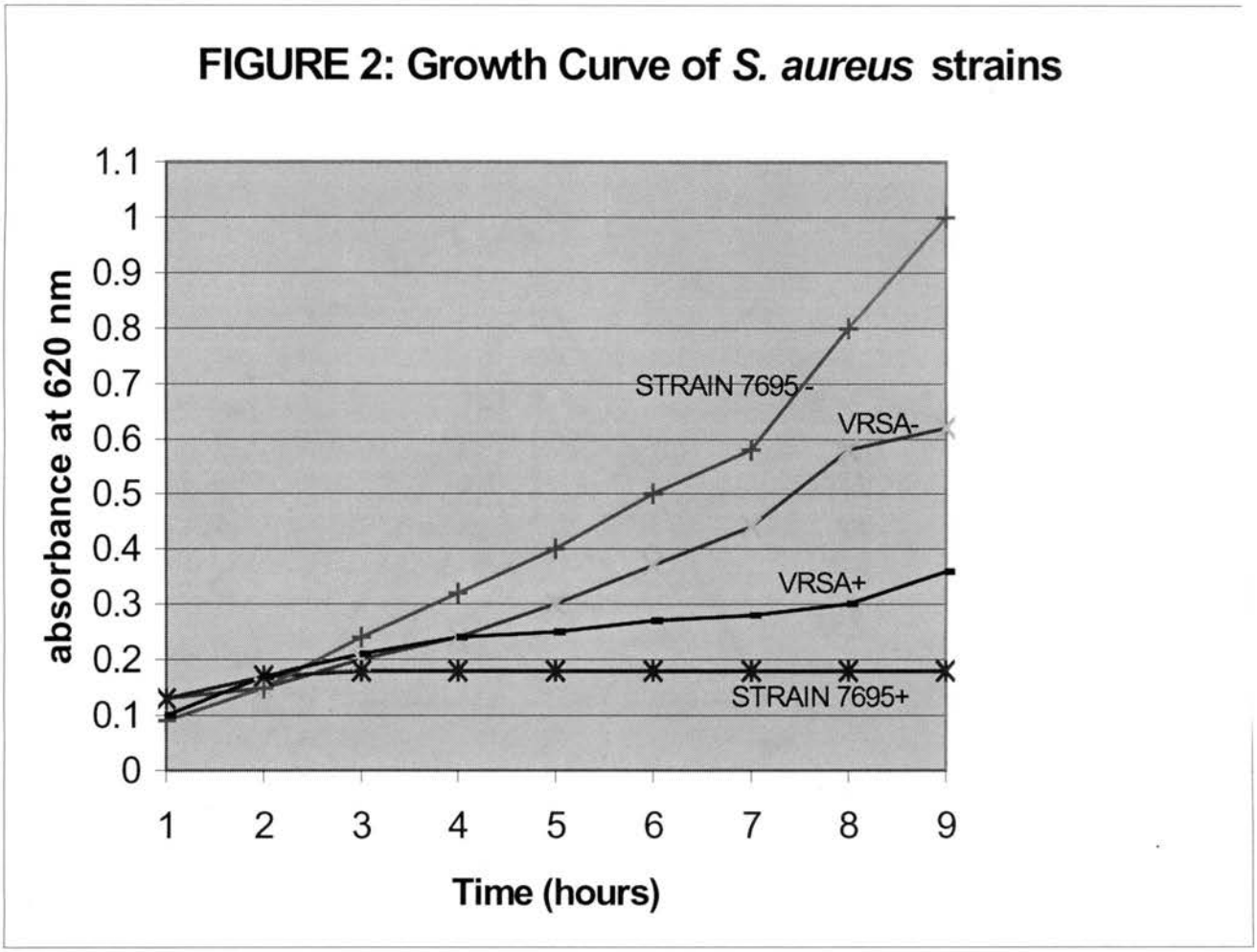
E.1. *S. aureus* strains:

The WTSA and strain 7695 decreased in turbidity from 0.60 to 0.05 within 60 min when cultured in the presence of lysostaphin. VRSA decreased in turbidity from 0.6 to 0.23 over the same amount of time (Figure 4). VRSA demonstrated increased resistance to lysostaphin by a 37% decline in turbidity as compared with the other *S.aureus* strains, which decreased by 92%.

E.2. *S. epidermidis* strains:

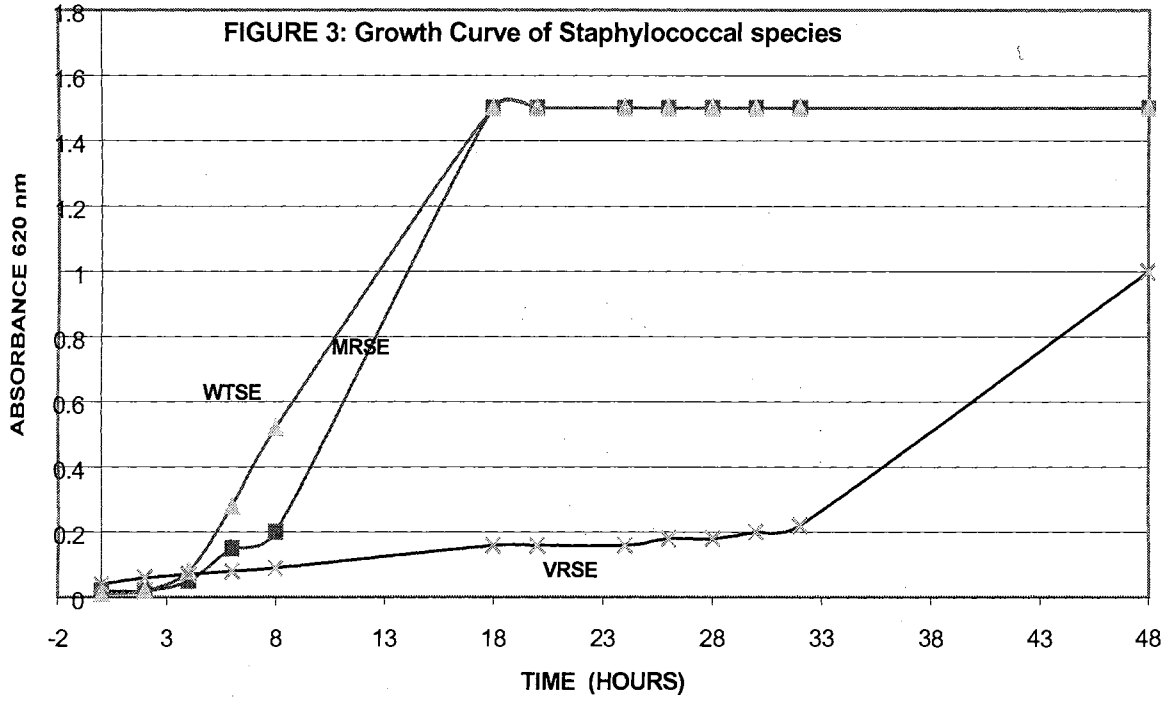
MRSE declined from 0.6 to 0.1 within 60 minutes of incubation with lysostaphin, a decline of 83%. VRSE declined from 0.45 to 0.40 within the same time period of

incubation, a decline of 11% (Figure 5).



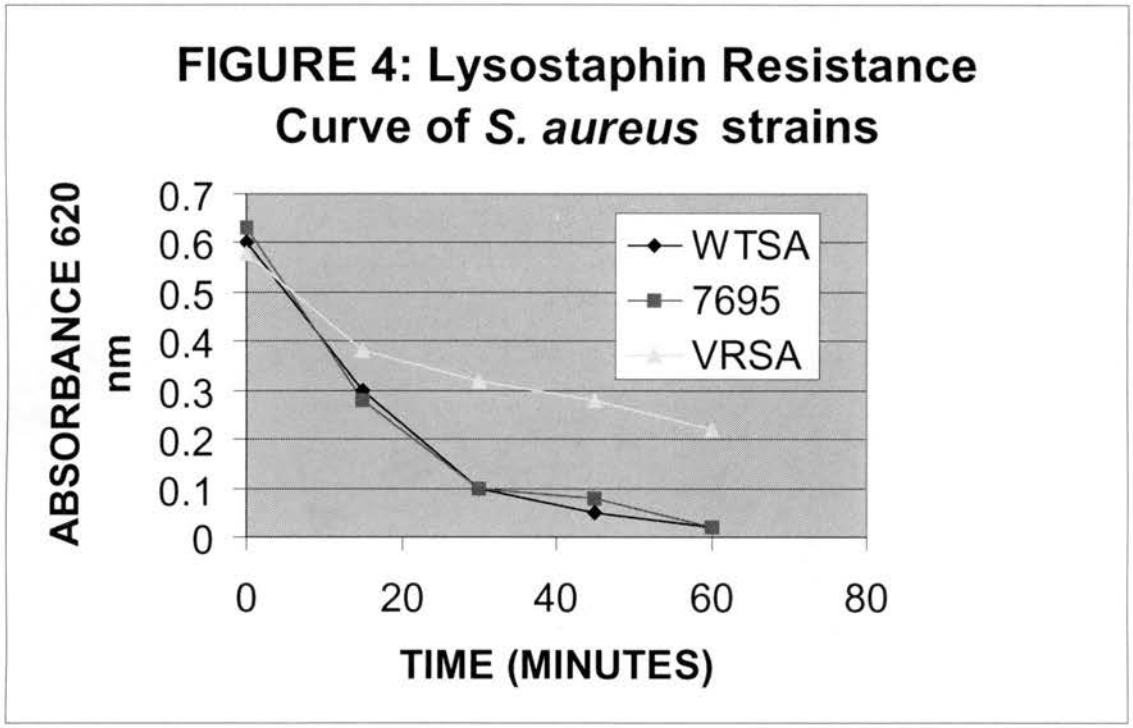
LEGEND FOR FIGURE 2:

Growth Curve for *S. aureus* strains: Strain 7695- and VRSA- grown in CSMH without vancomycin.
Strain 7695+ and VRSA+ grown in CSMH containing 32 µg/ml vancomycin.



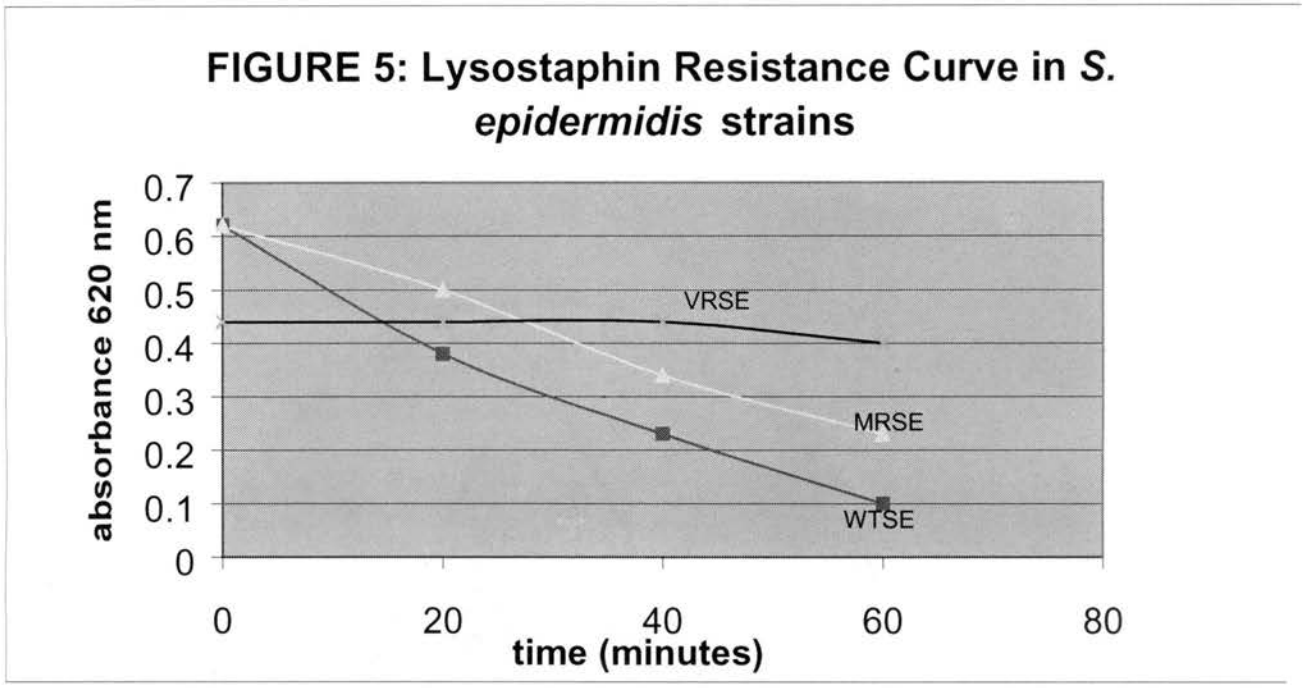
LEGEND FOR FIGURE 3:

Growth Curve for VRSE, MRSE and WTSE strains grown in CSMH without the addition of vancomycin.



LEGEND FOR FIGURE 4

Lysostaphin Resistance Curve of *S. aureus* strains: WTSA, STRAIN 7695 and VRSA grown in CSMH. Lysostaphin (1 unit/ml) added at time 0 to all cultures.



LEGEND FOR FIGURE 5:

Lysostaphin Resistance Curve of *S. epidermidis* strains: WTSE, VRSE and MRSE grown in CSMH. Lysostaphin (1unit/ml) added at time 0 to all cultures.

IV F Electron Microscopy

F.1. Scanning Electron Microscopy

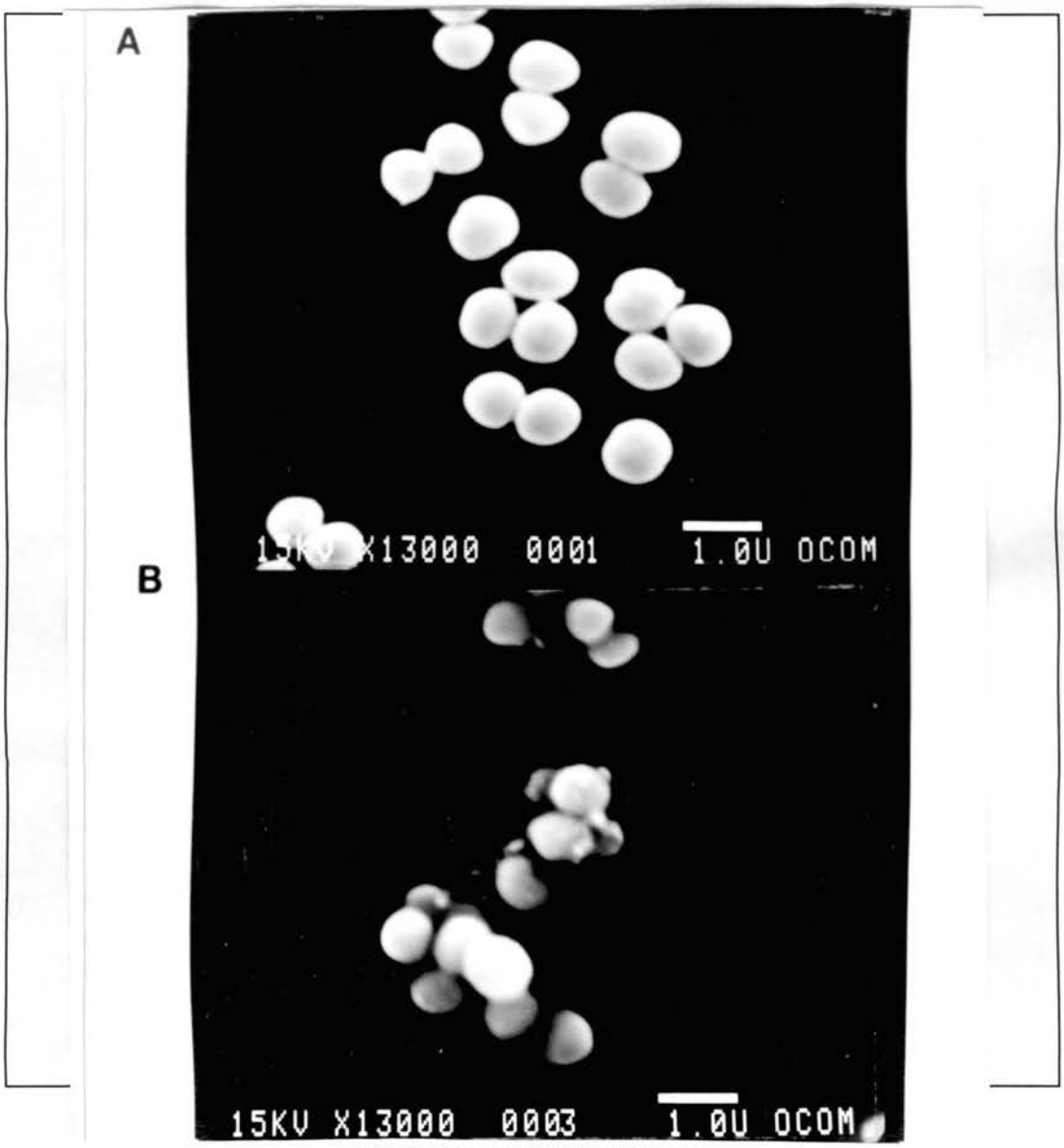
MRSE, strain 7695, VRSA and VRSE presented as grape like clusters typical of staphylococcal species when grown in the absence of vancomycin. MRSE and strain 7695 showed cytoplasmic streaming and cell wall damage when cultured with vancomycin (Figure 6,7). VRSA showed spherical cells and the external appearance of septa, but no cytoplasmic streaming or other evidences of cellular damage when grown in the presence (Figure 8).

VRSE also demonstrated increased septation when grown in the presence of vancomycin (Figure 9).

F.2. Transmission Electron Microscopy

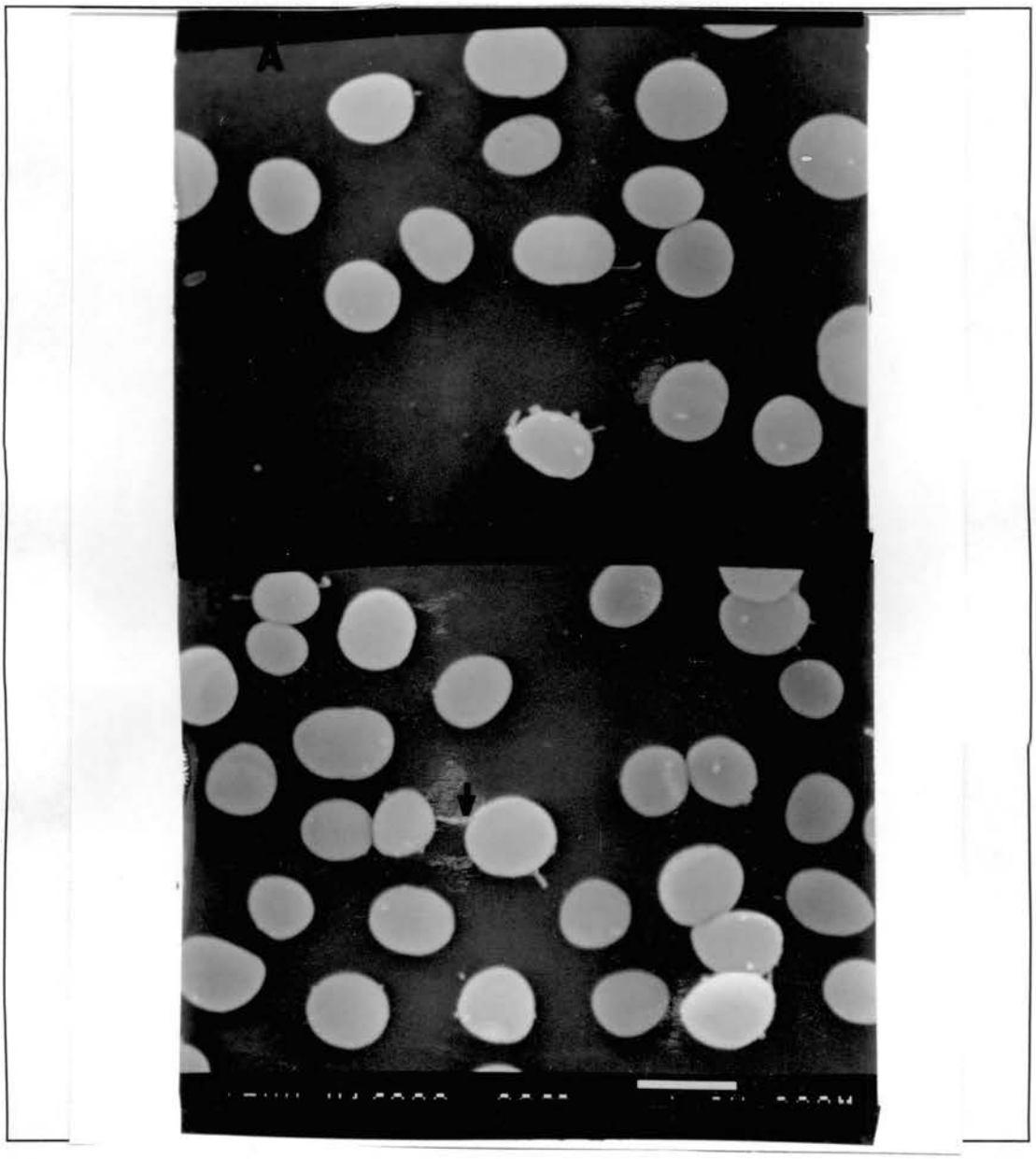
WTSA, WTSE, strain 7695 and MRSE appeared as spherical cells with no notable inclusions and normal cell wall thickness when grown in antimicrobial-free media. Growth in the presence of vancomycin produced evidence of damaged cell walls and ghost cells in susceptible strains, consistent with the scanning electron micrographs (Figure 10, 11, 12).

VRSA was grown in the absence of vancomycin and this isolate displayed thickened cell walls and a lack of cellular division as demonstrated by increased septation. VRSA, grown in the presence of vancomycin, showed increased cells with multiple septa, thickened cell walls and amorphous material adhering to the cell walls (Figure 11).



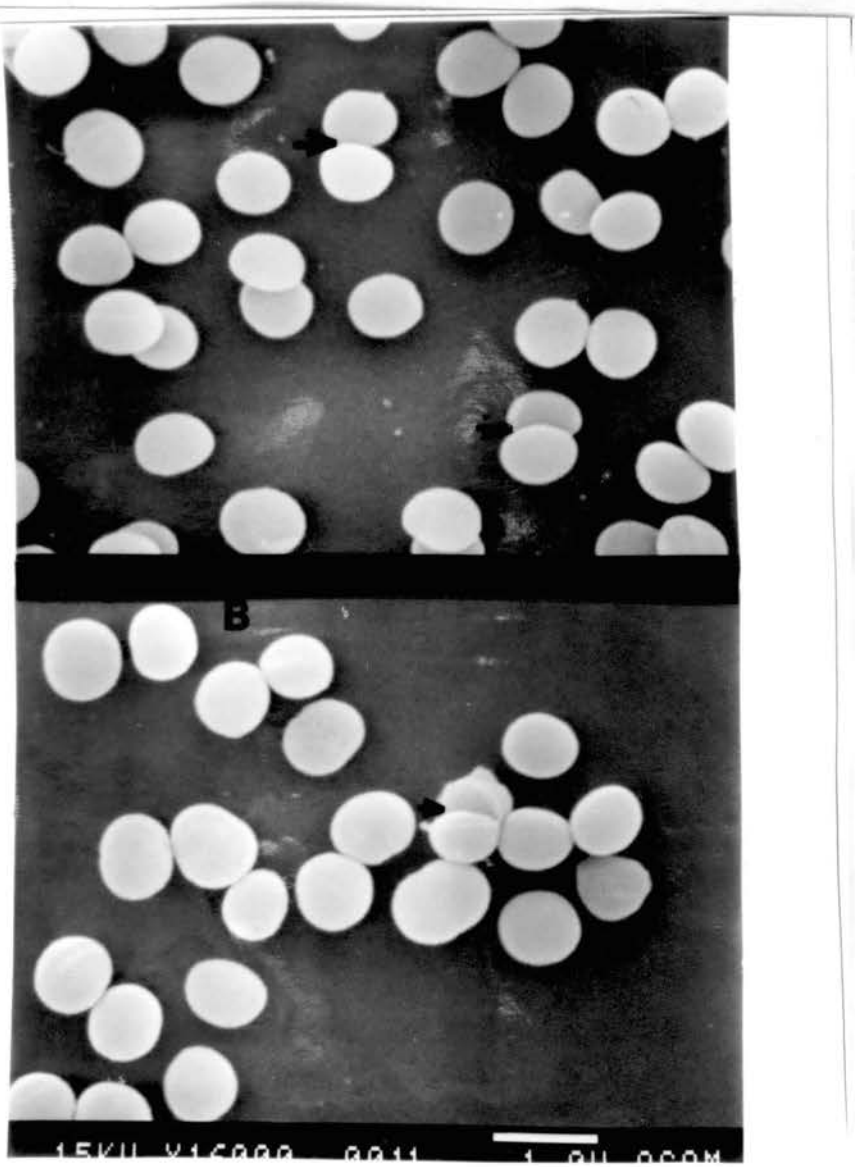
LEGEND FOR FIGURE 6:

SEM of MRSA:
A: MRSE grown in CSMH in the absence of vancomycin, 13,000 X
B: MRSE grown in CSMH, and then placed into fresh CSMH containing 32 µg/ml vancomycin for six hours. Arrow points to cytoplasmic streaming and cellular destruction. 13,000 X



LEGEND FOR FIGURE 7:

SEM of Strain 7695:
A: strain 7695 grown in CSMH containing no vancomycin. 16,000 X
B: strain 7695 grown in CSMH, and then placed into fresh CSMH containing 32 μg/ml vancomycin for six hours at 37°C. 16,000 X. Arrow denotes cellular blebs and partial cellular destruction.

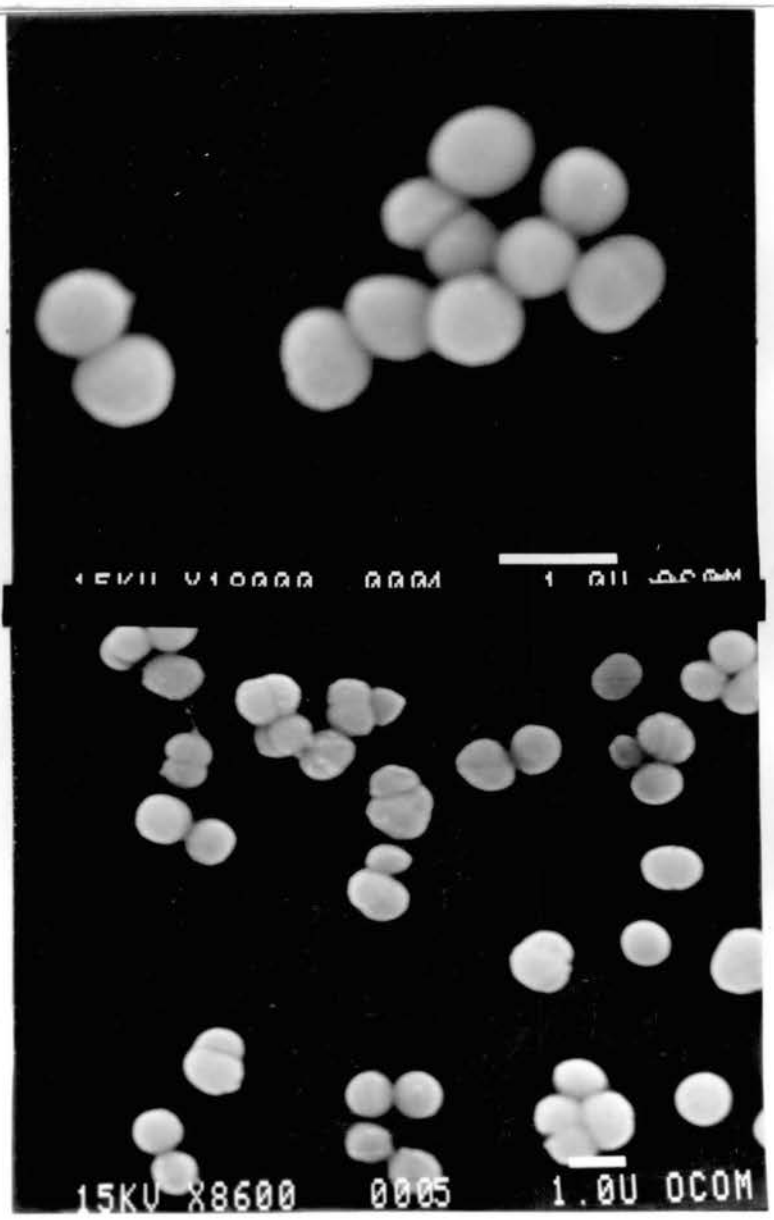


LEGEND FOR FIGURE 8:

SEM of VRSA

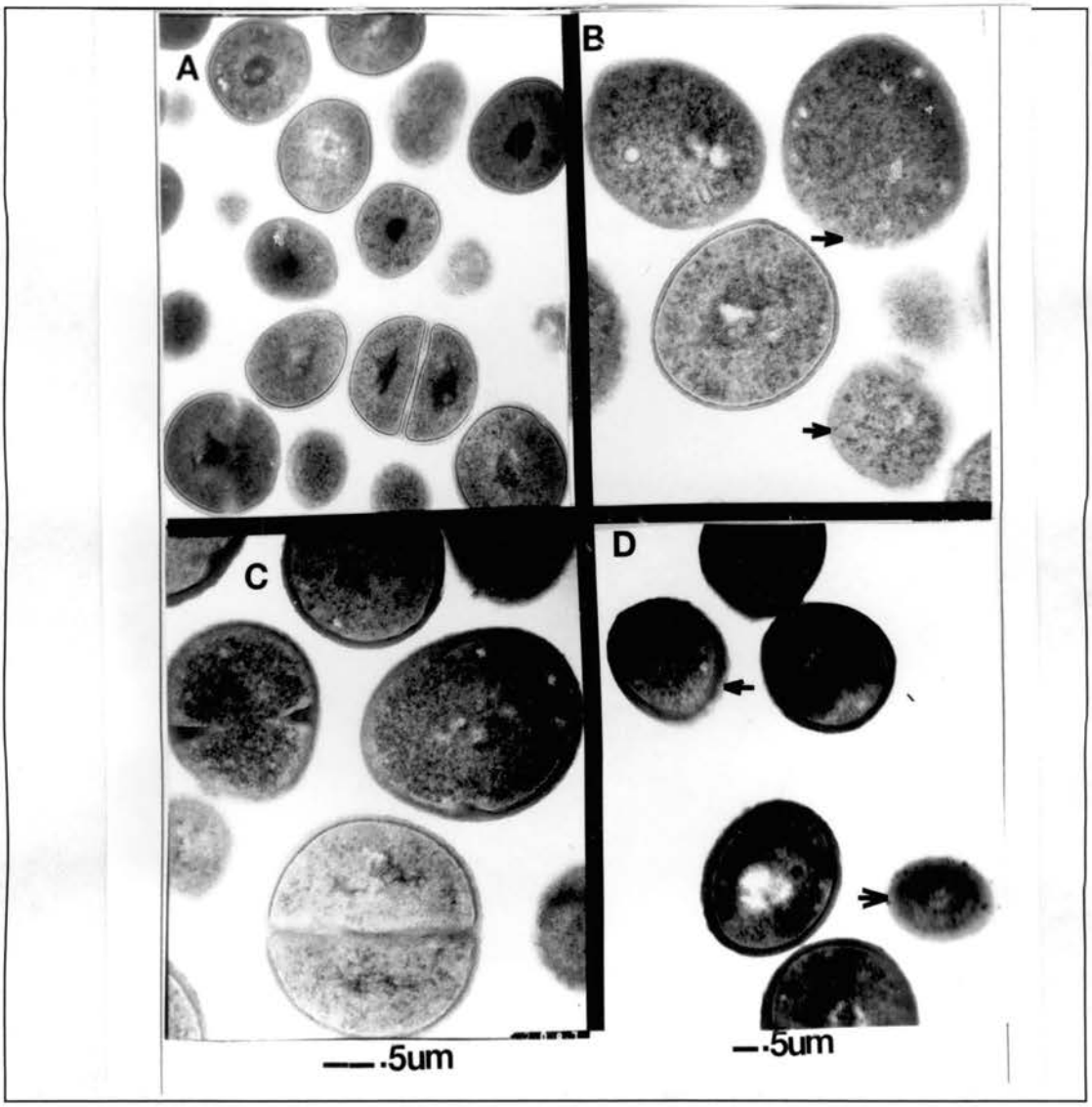
A: VRSA grown in CSMH containing no vancomycin, 16,000 X. Arrows denote increased number of cells with septation.

B: VRSA grown in CSMH, and then placed into fresh CSMH containing 32 μg/ml vancomycin for six hours at 37°C. 16,000 X. Arrows denote cells with abnormal septation.



LEGEND FOR FIGURE 9

SEM of VRSE
A: VRSE grown in CSMH without vancomycin, 18,000 X
B: VRSE grown in CSMH containing 32 μg/ml vancomycin, 8600 X. Note increased number of septated cells.



LEGEND FOR FIGURE 10:
TEM of WTSA and MRSE
A: WTSA grown in CSMH containing no vancomycin.20,000 X.
B: WTSA grown in CSMH, then placed into fresh CSMH containing 32 μg/ml vancomycin for six hours at 37°C.30,000 X. Arrows denote ghost cells and cell wall damage.
Scale = 0.5 micron (μm)
C: MRSE grown in CSMH containing no vancomycin, 30,000 X.
D: MRSE grown in CSMH, and then placed into fresh CSMH containing 32 μg/ml vancomycin for six hours at 37°C. 20,000 X. Arrows denote ghost cells and cell wall damage.
Scale = 0.5 micron (μm)

VRSE was grown in the absence of vancomycin and this isolate displayed thickened cell walls and lack of cellular division similar to the micrographs of VRSA. It is noteworthy that the VRSE cells also contained morula-like inclusions that were not readily demonstrated in the VRSA. VRSE grown in the presence of vancomycin appeared morphologically similar to VRSA with thickened cell walls, increased septation and amorphous material adhering to the cell wall (Figure 12). Cell wall measurements indicated that the cell walls of the VRSE and VRSA strains approximated twice the thickness of the susceptible strains.

IV G Cell Membrane Proteins Assays

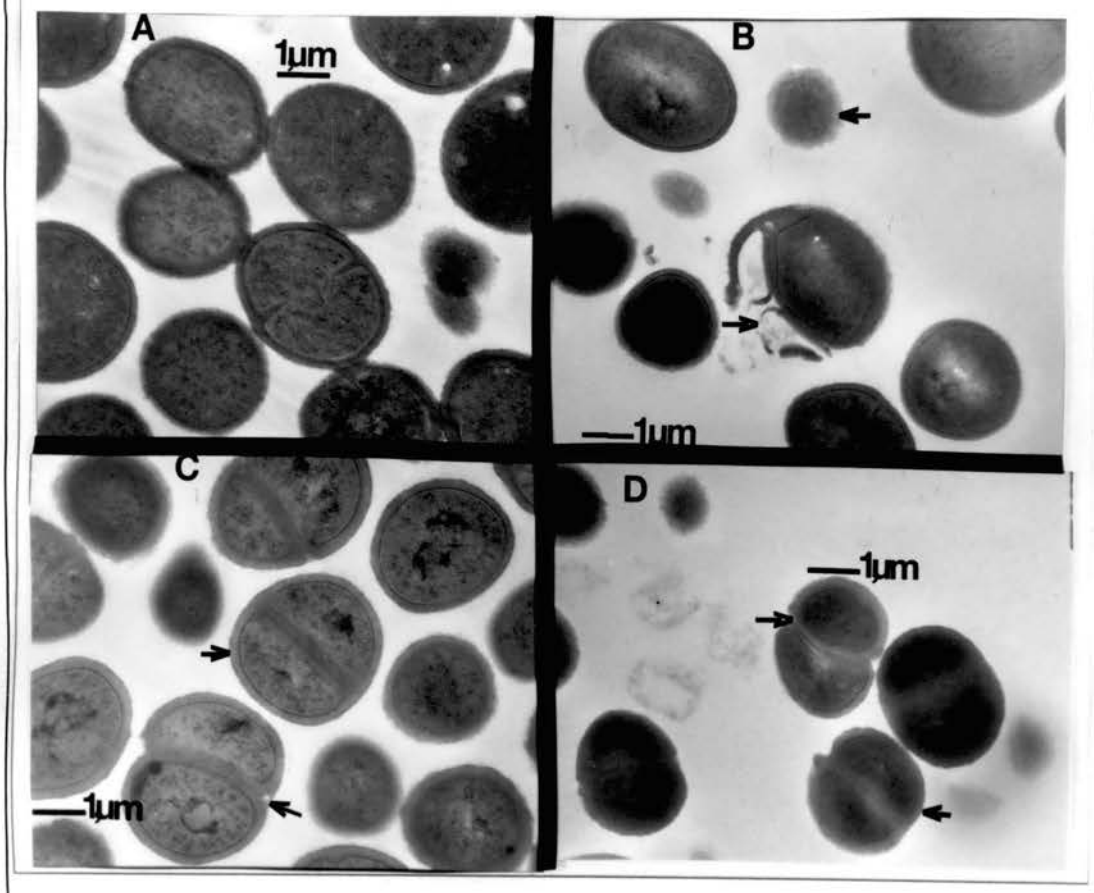
G.1. SDS-PAGE of *S. aureus* strains

Figure 13 is a photograph of the SDS-PAGE for *S. aureus* strains.
Lanes 3,5, 8 and 10 contain proteins that were treated with vancomycin. Lanes 2, 4, 7 and 9 contain proteins that were not treated with vancomycin. Lane 6 contains lysozyme (25,000 mw) and lysostaphin (35,000 mw) standards.
All staphylococcal strains contained intensely stained protein bands at 62,000 kD and 23,000 kD, as well as other common bands at 43,000 kD, 30,000 kD and 25,000 kD (lysozyme). These protein bands were of the same stained intensity, which provided visual evidence of similar protein concentration among samples.

VRSA had a decreased protein band at 52,000 kD compared with the other strains, and a deleted band at 48,000 kD. VRSA had a protein band at 28,000 kD that was not present in the other strains.

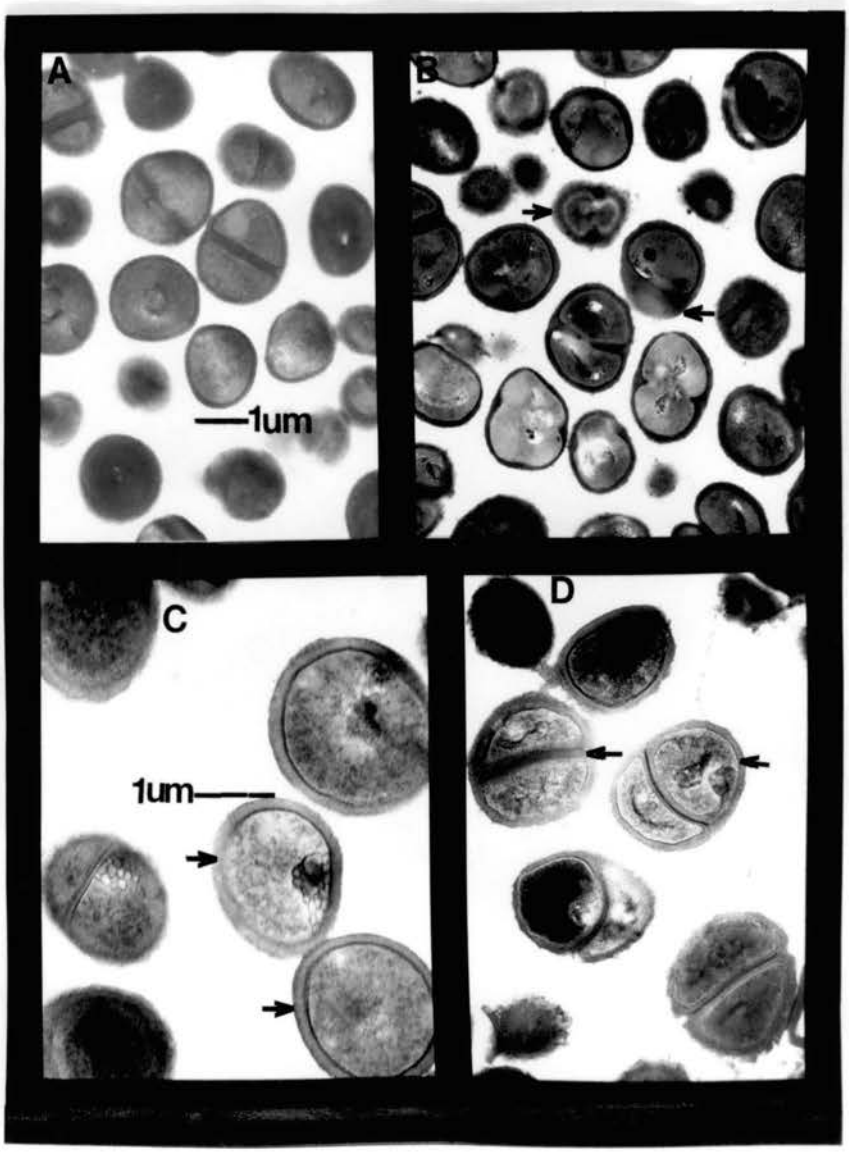
LEGEND FOR FIGURE 11:

TEM of Parent *S. aureus* and VRSA
A: Strain 7695 grown CSMH containing no vancomycin, 20,000 X.
B: Strain 7695 grown in CSMH, and then placed into fresh CSMH containing 32 μg/ml vancomycin for six hours at 37°C. 20,000 X. Arrows denote ghost cells and cell wall damage.
Scale = 1 micron (μm)
C: VRSA grown in CSMH containing no vancomycin, 20,000 X. Arrows denote increased cell wall thickness and increased septation.
D: VRSA grown in CSMH, and then placed into fresh CSMH containing 32μg/ml vancomycin for six hours at 37°C. 20,000 X. Arrows denote increased cell wall thickness, amorphous material adhering to cell wall periphery, and increased septation.
Scale= 1 micron (μm)


A
1µm
B
1µm
C
1µm
D
1µm

LEGEND FOR FIGURE 12:

TEM of WTSE and VRSE
A: WTSE grown in CSMH containing no vancomycin
B: WTSE grown in CSMH, and then placed into fresh CSMH containing 32 μg/ml vancomycin for six hours at 37°C.
C: VRSE grown in CSMH containing no vancomycin
D: VRSE grown in CSMH, and then placed into fresh CSMH containing 32 μg/ml vancomycin for six hours at 37°C.


A
—1um
B
C
1um——
D

The missing protein 48,000 was suspected to be PBP4 because of the similar molecular weight. Figure 14 is a photograph of the *S. aureus* membrane proteins labeled with fluorochrome-labeled penicillin. Two labeled bands are present in MRSA and strain 7695, but only one protein band is labeled in the VRSA. This assay shows that a penicillin-binding protein is missing from VRSA, but it does not identify which PBP is missing.

G.2. *S. epidermidis* strains

Figure 15 is a photograph of the SDS-PAGE of *S. epidermidis* and *S. aureus* strains. Lane 1 is a molecular weight standard. Lanes 2 and 3 are proteins from *S. aureus* strains and Lanes 4 and 5 are VRSE proteins. VRSE, grown in the presence of vancomycin, is missing a protein band at 48,000 kD. All isolates contained protein bands at 52,000 kD, 43,000 kD and 23,000 kD.

IV H Polymerase Chain Reactions

H.1. *S. aureus* strains

Five different PCR primer pairs were used to detect genes on the *S. aureus* genome. PCR products were detected in MRSA for *mecA*, *pbp4*, *blaz*, *coag* and *femB*. Strain 7695 was also positive for *mecA*, *pbp4*, *blaz*, *coag* and *femB*. VRSA was positive for *femB*, *coag* and *mecA* but negative for *blaz* and *pbp4*.

Figure 16 is a picture of the agarose gel of *S. aureus* strains for *mecA and pbp4*.

Figure 17A is a photograph of the agarose gel of the strains assayed for *femB*.

Figure 17B is an agarose gel photograph of *S. aureus* strains assayed for *coag*.

Figure 17C a photograph of the agarose gel of the *blaz*

Figure 17D is a photo of the agarose gel assay for *pbp4*.

H.2. *S. epidermidis* strains

Three different primer pairs were used to assay for *mecA, femB* and *pbp4* in S. *epidermidis* strains. Assays for *coag* and *blaz* were not done on these strains.

WTSE and VRSE were both was positive for *mecA*.

Figure 18A is a photograph of the *mecA* agarose gel.

The *S. epidermidis* strains were negative for *femB and pbp4*. Figure 18B is a photograph of the *femB* agarose gel.

Table IV is a summary of the PCR products detected in staphylococcal species.

TABLE IV **Summary of PCR Products**

PCR PRODUCT	WTSA	MRSA	7695	VRSA	WTSE	VRSE
Mec A	NEG	POS	POS	POS	NEG	**POS**
Coag	POS	POS	POS	POS	ND	ND
FemB	POS	POS	POS	POS	NEG	NEG
Pbp4	POS	POS	POS	**NEG**	NEG	**NEG**
Blaz	NEG	POS	POS	**NEG**	ND	ND

IV I Readily Extractable Lipids (REL)

Strain 7695 and VRSA were grown in antimicrobial-free media and evaluated for cell membrane fatty acids. VRSA was evaluated for fatty acids after growth in the presence of vancomycin. The most common membrane fatty acids found in Staphylococcal species are branched chain fatty acids with C15 or C17 carbon chains (98). Strain 7695 and VRSA were similar in the presence and quantity of expected staphylococcal lipids. The chromatography retention times included 100% of the fatty acids present in staphylococci. Strain 7695 contained 38% anteiso C15 (branched-chain) fatty acids, 23% anteiso C17(branched-chain) and 4% longer chain fatty acids of C20. VRSA grown in the antimicrobial-free media contained 35% branched-chain C15 fatty acids, 26% anteiso C17 and 4% C20 fatty acids. VRSA, grown in the presence of vancomycin, contained 26% anteiso C15, 14% anteiso C17 and had 19% C20 and longer fatty acid chains.

Table V is a summary of the fatty acid percentages in *S. aureus* strains.

TABLE V	**SUMMARY OF READILY EXTRACTABLE LIPIDS**				
Fatty Acid	**7695**	**VRSA**	**VRSA+**		
Branched-chain C15	38%	35%	26%		
Branched-chain C17	23%	26%	14%		
Long chain (>C20)	4%	5%	**19%**		
	VRSA+: Grown in the presence of 32 µg/ml vancomycin				

LEGEND FOR FIGURE 13: SDS-PAGE of *S. aureus* strains

Lane 1= VRSA with vancomycin

Lane 2= VRSA without vancomycin

Lane 3= MRSA with vancomycin

Lane 4= MRSA without vancomycin

Lane 5= Lysostaphin and Lysozyme standards

Lane 6= Strain 7695 with vancomycin

Lane 7= Strain 7695 without vancomycin

Lane 8= WTSA with vancomycin

Lane 9= WTSA without vancomycin

Lane 10 = Molecular weight standard

Arrow at 48,000kD: Note decreased to absent protein band in VRSA at this molecular weight.

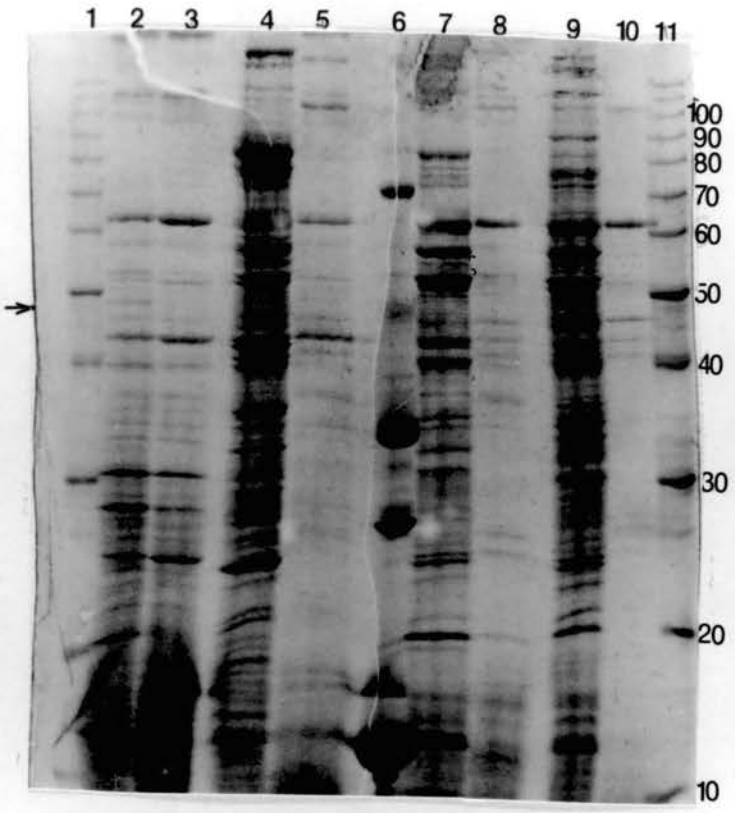


FIGURE 13

LEGEND FOR FIGURE 14: PENICILLIN BINDING PROTEINS OF *S.aureus*

Lane 1: Strain 7695

Lane 2: VRSA

Lane 3: MRSA

Lane 4: VRSA

Membrane proteins incubated with flourochrome-labelled penicillin (Bocillin) and separated on 10% native polyacrilamide gel containing no SDS. Proteins do not necessarily migrate by molecular weight on native gel. Note that the parent S. aureus and MRSA both contain two large bands that bind labeled penicillin. VRSA has only one band that binds the labeled penicillin.

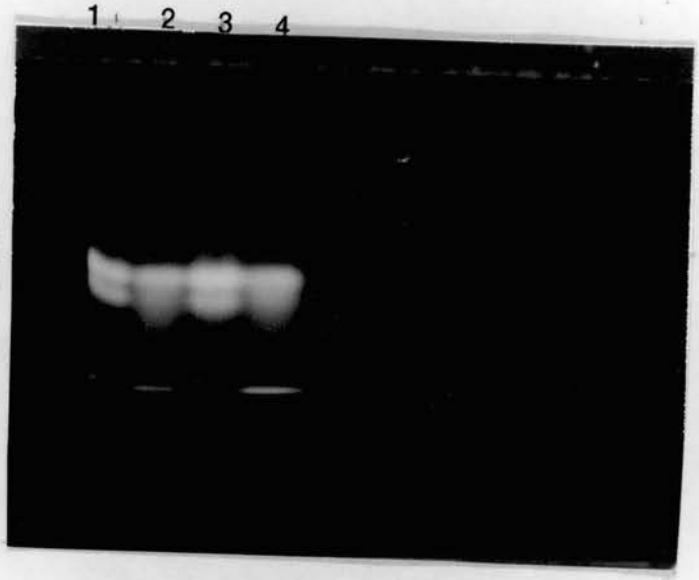


FIGURE 14

LEGEND FOR FIGURE 15: SDS-PAGE of VRSE

Lane 1= Molecular weight standard

Lane 2=WTSA

Lane 3=MRSA

Lane 4=VRSE without vancomycin

Lane 5= VRSE with vancomycin

Lane 6= Lysostaphin and lysozyme standards

Lane 7= Molecular weight standard

Lane 8= WTSA

Lane 9= MRSA

Lane 10= VRSE without vancomycin

Lane 11= VRSE with vancomycin

Lane 12= Lysostaphin and lysozyme standard

Note: missing protein band in lanes 5 and 11 at 48,000kD

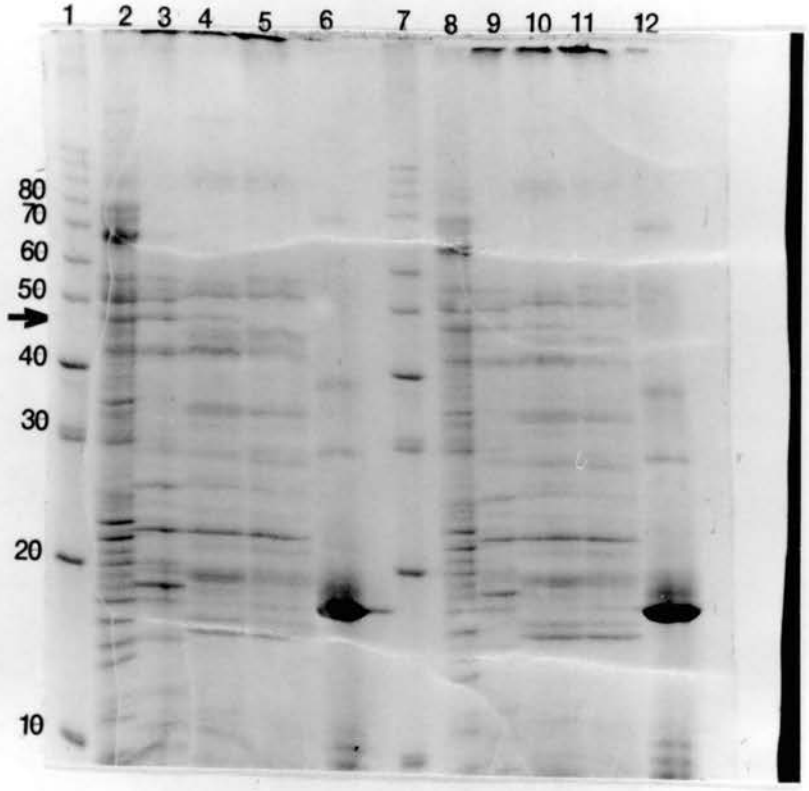


FIGURE 15

LEGEND FOR FIGURE 16

POLYMERASE CHAIN REACTION OF *S.aureus* STRAINS

mecA (449bp) and *pbp4* (2000bp) products

Lane 1 = 100bp DNA ladder

Lane 2= MRSA (*pbp4)*

Lane 3= MRSA (*mecA*)

Lane 4= 7695 (*pbp4 and mecA*)

Lane 5= VRSA (*pbp4* and *mecA*)

Lane 6= MRSA (pbp4)

Lane 7= Primer control (negative)

Lane 8= no DNA added

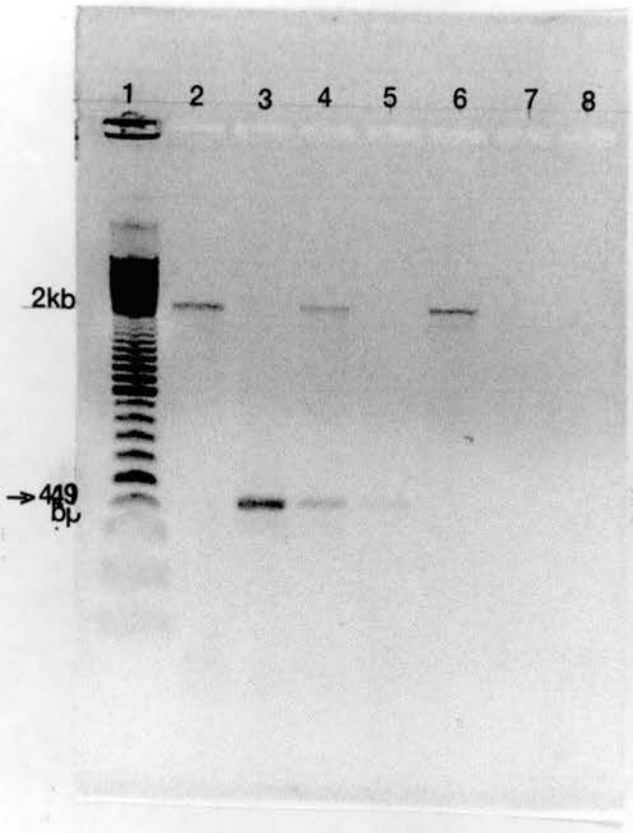


FIGURE 16

LEGEND FOR FIGURE 17

POLYMERASE CHAIN REACTION OF *S.aureus* STRAINS

Fig. 17A- *blaz* gene (2000bp)

Lane 1=100 bp DNA ladder
Lane 2=MRSA
Lane 3=Strain 7695
Lane 4=VRSA
Lane 5=VRSA grown in vancomycin
Lane 6=WTSA
Lane 7=MRSA
Lane 8= Primer control

Fig.17B-*femB* gene (651bp)

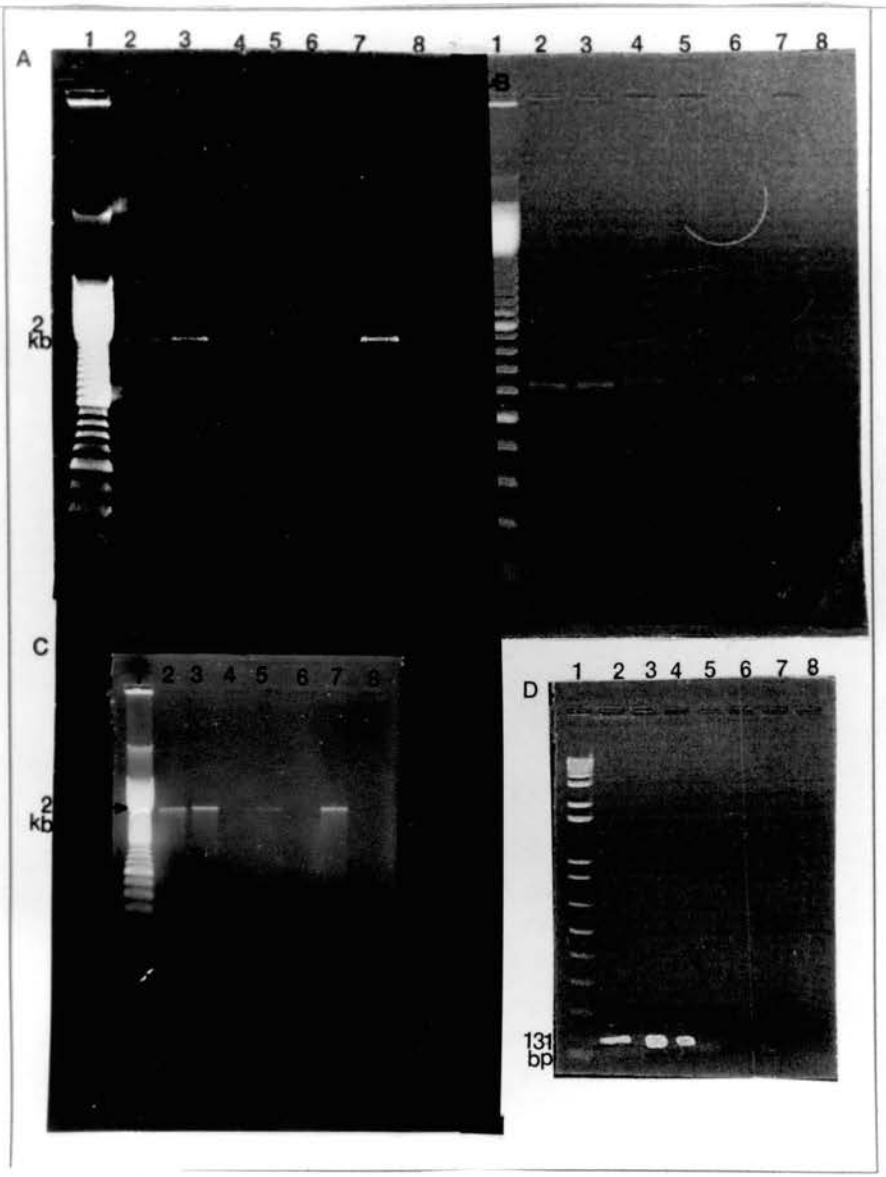
Lane 1= 100 bp DNA ladder
Lane 2= MRSA
Lane 3= Strain 7695
Lane 4= VRSA
Lane 5= VRSA grown in vancomycin
Lane 6= MRSA
Lane 7= primer control
Lane 8= no DNA added

Fig. 17C- *pbp4* gene (2000bp)

Lane 1=100 bp DNA ladder
Lane 2=MRSA
Lane 3= Strain 7695
Lane 4=VRSA
Lane 5= Strain 7695
Lane 6= VRSA
Lane 7= MRSA
Lane 8= Primer control

Fig. 17D-*coag* gene (131 bp)

Lane 1= 100 bp DNA ladder
Lane 2= MRSA
Lane 3= Strain 7695
Lane 4=VRSA
Lane 5=WTSE
Lane 6=VRSE
Lane 7= Primer control
Lane 8= no DNA added


A
1 2 3 4 5 6 7 8
2 kb
1 2 3 4 5 6 7 8
C
2 3 4 5 6 7 8
2 kb
D
1 2 3 4 5 6 7 8
131 bp

LEGEND FOR FIGURE 18

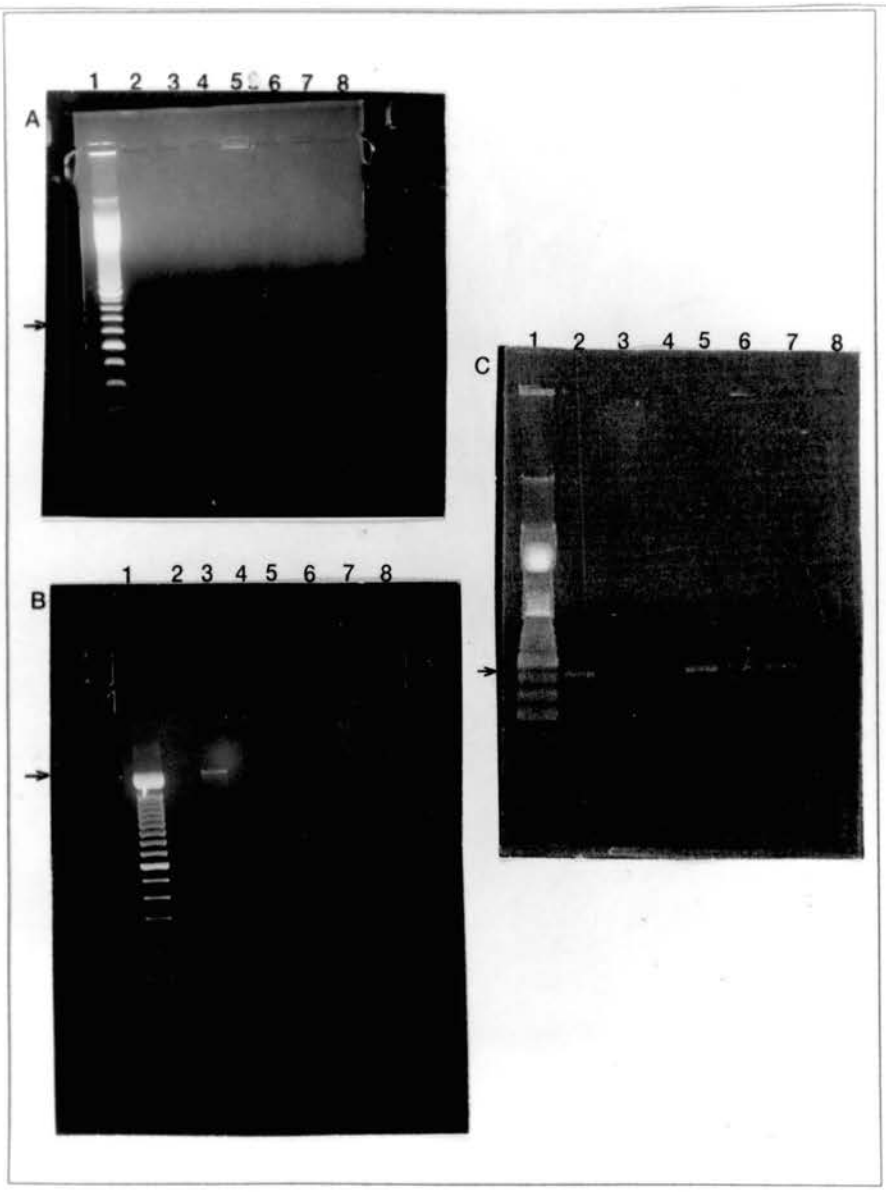
PCR PRODUCTS OF *S. epidermidis* strains

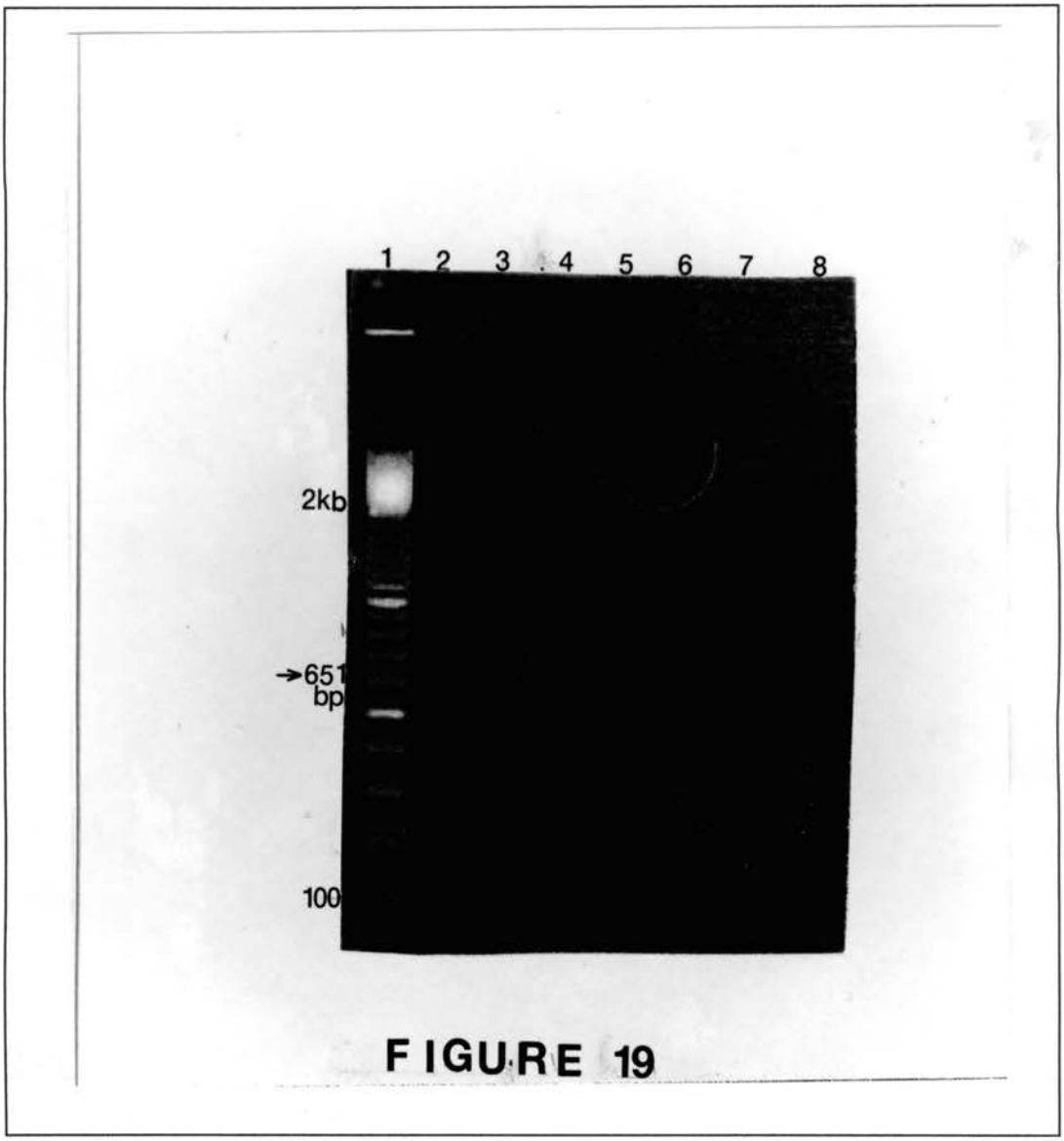
18A: *mecA*

Lane 1: 100bp ladder
Lane 2: MRSA (positive control)
Lane 3: strain 7695
Lane 4: VRSE
Lane 5: WTSE
Lane 6: WTSE
Lane 7: VRSE
Lane 8: primer control

18 B: *pbp4*
Lane 1: 100 bp ladder
Lane 2: VRSE
Lane 3: WTSE
Lane 4: VRSE
Lane 5:empty
Lane 6: empty
Lane 7: primer control

18 C: *femB*
Lane 1: 100bp ladder
Lane 2: MRSA(positive control)
Lane 3: VRSE
Lane 4: WTSE
Lane 5: MRSA
Lane 6: VRSE
Lane 7: strain 7695
Lane 8: primer control


A
1 2 3 4 5 6 7 8
B
1 2 3 4 5 6 7 8
C
1 2 3 4 5 6 7 8



LEGEND FOR FIGURE 19: PCR of *femB*, all strains

Lane 1= MRSA
Lane 2=7695
Lane 3=VRSA
Lane 4=no DNA
Lane 5=WTSE
Lane 6=VRSA
Lane 7=Primer control

CHAPTER FIVE

V SUMMARY AND DISCUSSION

V A Summary of Research Findings

A.1. Adaptation

Staphylococcal clinical isolates of varying antimicrobial susceptibilities grew in the presence of sub-lethal concentrations of vancomycin. The isolates were transferred into increasing vancomycin concentrations and many of these isolates were unable to survive at vancomycin concentrations of 16 μg/ml or greater.

Two of the isolates, strains 7695 and MRSE, adapted to vancomycin concentrations of 32μg/ml although the adaptation process took several months to achieve stable consistent growth. In response to long- term exposure to vancomycin, the adapted strains referred to as VRSA and VRSE, altered their growth patterns, cell wall composition, protein synthesis, phenotype and genotype. The alterations were considered to be stable and permanent when assays performed before and after all strains had been frozen at –20°C for four months yielded identical results.

A.2. Growth and morphology changes

Both adapted strains grew more slowly than the original isolates. In both strains the generation times were almost double that of the original isolate. Both strains had greater resistance to lysostaphin than the original strains, which were completely degraded by the endopeptidase.

Strains 7695 and MRSE displayed normal staphylococcal morphology of grape-like cluster when grown in antimicrobial-free media. Both isolates showed cell wall damage when grown in the presence of vancomycin. In contrast, VRSA and VRSE exhibited thickened cell walls and an increase in the number of septated cells regardless of growth conditions. When the adapted strains were grown in the presence of vancomycin, amorphous material was noted adhering to the periphery of the cell wall. Diameter measurements of the adapted strain cell walls were almost twice the measurements of the original strain cell walls.

VRSA synthesized an increased quantity of longer chain cell membrane fatty acids when grown in vancomycin-containing media. The quantity and length of the cell membrane fatty acids were identical to the original isolate when VRSA was grown in antimicrobial-free media.

A.3. Antimicrobial susceptibilities

Strains 7695 and MRSE were initially susceptible to vancomycin and resistant to B-lactam antimicrobials. Strain 7695 was susceptible to methicillin until the isolate was exposed to the antimicrobial. Growth in sub-lethal concentrations of methicillin resulted in the strain becoming resistant to the drug. Both strains were similar in susceptibility profiles to other antimicrobials tested.

Upon completion of the adaptation process, MIC assays revealed that both adapted strains measured in the range for vancomycin resistance. Conversely, VRSE and VRSA lost resistance to B-lactam antimicrobials. When MICs of original and adapted strains were compared, no other changes were noted in the antimicrobial susceptibility patterns.

A.4. Phenotypic changes

Results of assays for coagulase, β-lactamase and protein A were positive for Strain 7695. The strain adapted from strain 7695, VRSA, was negative for β-lactamase and the coagulase assay required 24 hours for a positive result. None of these assays were performed for the *S. epidermidis* strains.

Carbohydrate fermentation studies revealed no inconsistent patterns. The original clinical isolate of MRSA did not ferment mannitol, nor did the strain adapted from MRSE, VRSE.

A.5. Cell membrane protein changes

A cell membrane protein of 48kD was deleted in both VRSA and VRSE. This protein was present in the original strains as well as wild-type staphylococci. PBP-labeling studies showed that VRSA was missing a PBP as compared to the original strain, but it could not be concluded that the PBP was the deleted 48kD cell membrane protein. Both adapted strains synthesized a protein of 28kD that was not found in the other strains.

A.6. Genotypic changes

Strain 7695, and MRSA displayed PCR products for *femB, pbp4, coag, blaz* and *mecA* genes. In contrast, VRSA did not have PCR products for *pbp4, or blaz* but did have products for *femb, mecA* and *coag.*

VRSE was positive for products to *mecA*, but not for *femB* or *pbp4.* The genes for *coag* and *blaz* were not assayed for this strain.

VB Discussion

Exposure of staphylococcal isolates to vancomycin over a four-month period resulted in either lethal cellular damage or to numerous alterations in the strains that survived. The isolates grew more slowly and obtained thicker cell walls. The prolonged generation times reflect increase in cell wall synthesis and are evidenced by the thicker cell walls. Previous studies grew staphylococcal isolates grown in the presence of radio-labeled N-acetyl glucosamine and determined that adapted strains take up more cell wall precursors, but release the cell wall components more slowly (88). This study suggests that adapted strains increase in cell wall synthesis and decrease in cell wall autolysis (79). Cell wall autolysis allows aging cell wall components to be replaced with newly synthesized precursors to provide the cell with the space needed to grow (90). The presence of thicker cell walls, without a concomittent increase in total cell diameter might suggest a decrease in cell autolysis with simultaneous cell wall synthesis.

Lysostaphin resistance suggests that amino acid substitutions, such as serine, may have occurred in the cross bridge. *S. epidermidis* strains are innately more resistant to lysostaphin because serine is present at position #4 in the cross-bridge. Both VRSA and VRSE were more resistant to lysostaphin than original isolates and wild type strains. The protein FemB attaches adjacent glycines at position #4 and #5 in the cross-bridge. VRSA was positive for the *femB* gene but it was not determined if the protein was correctly translated. If the FemB protein was functional, then serine could not be present in position #4 of the VRSA cross-bridge, but could be present at another location. *S. epidermidis* strains do not have the gene for *femB,* nor is it required because serine replaces glycine in the #4 position.

Both adapted strains were resistant to vancomycin, but both lost resistance to methicillin. The genes for *blaz* was deleted or mutated in VRSA and the enzymes necessary for resistance to penicillin and ampicillin were not available. VRSA is also susceptible to methicillin, although the *mecA* gene was intact. It is possible that the protein product, PBP2a, is not translated or is mutated or that the deletion of the blaz gene contributes to the loss of methicillin resistance. It is curious that a microorganism would trade the ability to survive in the presence of one antimicrobial class in order coexist in the presence of another. Whether or not genetic mutations or deletions allow the activation of other genes to be expressed may contribute to the understanding of this phenomenon. Repeated exposure of the adapted strains to antimicrobial free media did not reverse the antimicrobial profile. VRSE did not lose the *mecA* gene but it was nevertheless susceptible to β-lactam antimicrobials. It is unknown if PBP2a was not translated or if other alterations resulted in the loss of resistance.

Although VRSA did possess the *coag* gene, the coagulase protein appeared to be transported more slowly through the thickened cell wall. Other factors could contribute to the prolonged release including cell membrane alterations in the adapted strain. Cell membrane fatty acids were increased in chain length as well as in quantity as compared to the original strain. It is possible the increased length of the fatty acids could block or delay the secretion of cytoplasmic proteins. Increased fatty acid chain length could also contribute to the delayed growth rate due to a steric hinderance of the transport of the cell wall precursors.

The adapted strains were missing a 48kD cell membrane protein. The molecular weight is identical to the molecular weight of PBP4. Penicillin binding studies showed that a PBP is missing from the VRSA, but proteins separated on native gels do not necessarily travel according to molecular weight. Neither VRSA nor VRSE possess the gene for *pbp4,* so it is possible that the missing protein is PBP4. PBP4 functions as a cell wall synthesis enzyme to remove the terminal D-alanine from the pentapeptide allowing the cross-linkage of L-lysine and D-alanine through the pentaglycine bridge (32,36). If PBP4 is inactivated, it is possible that the terminal D-alanine is not released resulting in a D-alanyl-D-alanine dipeptide in the terminus of the pentapeptide. This dipeptide is the site of vancomycin binding and may be binding vancomycin to the cell wall exterior. The attachment of vancomycin to the cell wall periphery may impede the transport of vancomycin to the interior of the cell where it could inhibit the synthesis of new cell wall components (28). Cross-linkage is compromised when the terminal D-alanine is not released requiring the cell wall to increase in synthesis to provide a stable complex.

A cell membrane protein of 28kD was unique to the adapted strains. Although the function of this protein was not investigated, it is possible this protein is necessary for cell wall synthesis in the absence of PBP4.

The mechanism of vancomycin resistance in staphylococci appears to involve genetic mutations that allow cell wall synthesis to proceed to circumvent the presence of a cell wall inhibitor. The adapted strains appear to trade resistance of one antimicrobial for another. Inactivation of PBP4 results in pentapeptide alterations that provide excess binding sites for vancomycin. Premature binding of vancomycin to the periphery of the

cell wall may prevent the drug from being transported internally where cell wall synthesis is occurring.

VC Suggestions for Areas of Further Research

In order to further delineate the mechanism of vancomycin resistance in *Staphylococcus,* the following studies could be performed:

1. Compare the DNA sequences of intact *pbp4* in the susceptible strains with the analogous DNA in vancomycin resistant strains.
2. Transform DNA of vancomycin susceptible strain into vancomycin resistant cells and examine the transformants for resistance to methicillin and susceptibility to vancomycin.
3. Expose VRSA and VRSE to sub lethal concentrations of methicillin in order to restore methicillin resistance.
4. Vancomycin binding studies of susceptible and resistant strains to determine if vancomycin binds to the cell wall, and if so, does that binding hinder the transport of vancomycin internally.
5. Determine the function of the 28kD cell membrane protein produced by the vancomycin resistant strains.
6. Label cell membrane proteins with fluorescent bound penicillin and determine if the missing 48kD protein is a PBP.

7. Analyze the amino acid composition of the peptidoglycan of the vancomycin susceptible and resistant strains. Determine if D-alanyl-D-peptides are present in either strain or if serine has been substituted into the cross bridge.
8. Determine if the gene for *femC* is present in the susceptible and resistant strains.

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Doctor of Philosophy

Thesis: MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* AND *STAPHYLOCOCCUS EPIDERMIDIS* STRAINS STEP-WISE ADAPTED TO VANCOMYCIN

Major Field: Biomedical Sciences

Biographical:

Education: Bethany Nazarene College (Southern Nazarene University); Bethany, Oklahoma. Bachelor of Science, 1973.
Hillcrest School of Medical Technology; Tulsa, Oklahoma. Medical Technologist,ASCP registered 1973.
California State University; Fullerton, California.1978-1979.
San Francisco State University; San Francisco, California. Master of Science, 1981.
University of Oklahoma; Oklahoma City, Oklahoma.1985-1986.
Oklahoma State University College of Osteopathic Medicine; Tulsa, Oklahoma.
Completed the Requirements for the Doctor of Philosophy degree in May 2001.

Experience: Medical Technologist (MTASCP) 1973-1991. Assistant Professor, Tulsa Community College, 1991-2001.

Professional Memberships: American Society of Microbiology
Oklahoma Academy of Science American Society of Clinical Pathologists